

Department of Physiology, Anatomy and Genetics

Doctor of Philosophy Thesis

The effects of an exogenously-induced ketosis on human exercise physiology, metabolism and capacity

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Abstract

Ketosis is a pivotal metabolic adaptation to starvation that prolongs survival by altering fuel utilisation. If harnessed outside the context of starvation, ketosis may provide a method to enhance physical performance. Therefore, this Thesis explored the effects of an exogenously-induced ketosis on human exercise physiology, metabolism and capacity. First, how the mild-ketoacidaemia associated with ketone supplementation affects the physiological response to incremental-intensity exercise and athletes' perceptions of exercise exertion were determined (Chapter 3). To compensate for ketoacidaemia, exercise ventilation was increased by 5% and exercise-induced hyperventilation was at a 20 W lower intensity. Perceptions of exertion were greater during ketosis, primarily due to the increased acid load. Chapter 4 determined the effects of blood β-hydroxybutyrate (βHB) concentration and exercise intensity on βHB oxidation rates. Increasing blood βHB concentration from $\approx$2 mM to $\approx$4.5 mM did not affect βHB oxidation rates. βHB oxidation rates peaked during 50% maximal power (W_{Max}) intensity exercise, although the greatest relative contribution to overall energy expenditure was during 25% W_{Max} intensity exercise and just 4.5%. Despite this, delta efficiency was increased during ketosis. Finally, in Chapter 5, the effects of prolonged exogenously- and endogenously-induced ketoses on exercise capacity and adaptive responses to exercise training were compared. Ketone supplementation improved exercise capacity by 4 - 6% on 50% of training days versus a carbohydrate (CHO) control, but markedly impaired whole-body insulin sensitivity. Conversely, a ketogenic diet substantially increased fat oxidation by $\approx$60% during exercise. In part, this was caused by changes in skeletal muscle protein content, most notably a 5-fold increase in pyruvate dehydrogenase kinase 4. However, exercise capacity was decreased by 41 - 48%. In conclusion, despite increased awareness of exercise exertion, and low βHB oxidation rates, supplementing athletes with ketones may enhance exercise efficiency and capacity in athletes. However, prolonged supplementation during exercise training may result in unfavourable metabolic adaptations to CHO metabolism, without reciprocal increases in fat metabolism, as observed with an endogenously-induced ketosis.

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Acronyms

1,3-BPG	1,3-bisphosphoglycerate	22
^{12}C	carbon 12	93
$^{12}CO_2$	carbon 12 labelled CO_2	93
^{13}C	carbon 13	89
$^{13}CO_2$	carbon 13 labelled CO_2	93
AcAc	acetoacetate	26
AcAc-CoA	acetoacetyl-CoA	28
ACAT1	acetyl-CoA acetyltransferase 1	29
Ac-CoA	acetyl-coenzyme A	22
acyl-CoAs	acyl-coenzyme As	23
ADP	adenosine diphosphate	23
ANOVA	analysis of variance	71
AUC	area under the curve	105
ATP	adenosine triphosphate	20
BDH	β-hydroxybutyrate dehydrogenase	31
βHB	β-hydroxybutyrate	1
BPM	beats per min	52
cAMP	cyclic adenosine monophosphate	126
cDNA	complementary DNA	63
CHO	carbohydrate	1
CO_2	carbon dioxide	30
CoA	coenzyme A	147
CPT I	carnitine palmitoyltransferase I	24
CrP	creatine phosphate	21
CS	citrate synthase	27
DALDA	daily analysis of life demands of athletes	116

DAPI	4',6-diamidino-2-phnylindole dihydrochloride	57
DGAT2	diacylglycerol O-acyltransferase 2	126
DNA	deoxyribonucleic acid	58
DNL	*de novo* lipogenesis	28
DPAG	Department of Physiology, Anatomy and Genetics	68
DTT	1,4-Dithiothreitol	60
ELISA	enzyme-linked immunosorbent assay	70
F6P	fructose 6-phosphate	141
$FADH_2$	oxidised flavin adenine dinucleotide	23
FBLA	fixed blood lactate accumulation	43
FDA	Food and Drug Administration	51
FDR	false discovery rate	62
FITC	fluorescein isothiocyanate	57
FFA	free fatty acid	23
FFAR3	free fatty acid receptor 3	36
G3P	glyceraldehyde 3-phosphate	22
G6P	glucose 6 phosphate	21
GLMER	general linear mixed effect regression	72
GLUT 1	solute carrier family 2, facilitated glucose transporter member 1	150
GLUT 4	solute carrier family 2, facilitated glucose transporter member 4	126
H^+	proton	22
Hb	haemoglobin	69
HCAR2	hydroxycarboxylic acid receptor 2	35
HCl	hydrochloric acid	60
HCO_3^-	bicarbonate	41
HDAC	histone deacetylase	46
HMGCL	hydroxymethylglutaryl-CoA lyase	27
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA	27
HOMA IR	homeostatic model assessment of insulin resistance	131
HR	heart rate	36
HSL	hormone sensitive lipase	126

K^+	potassium	75
kcal	kilo calories	44
LDH	lactate dehydrogenase	35
MCT	monocarboxylic acid transporter	27
MEF2	myocyte enhancer factor 2	46
MPC	mitochondrial pyruvate carrier	27
MS	mass spectrometry	59
mTORC1	mammalian target of rapamycin complex 1	46
NAD^+	oxidised nicotinamide adenine dinucleotide	22
NADH	reduced nicotinamide adenine dinucleotide	21
NEFA	non-esterified fatty acids	70
NH_4	ammonium	35
O_2	oxygen	32
OCT	optimal cutting temperature	57
OGTT	oral glucose tolerance test	116
ORO	oil red o	62
OXREC	Oxford Research Ethics Review Committee	50
PCO_2	partial pressure of carbon dioxide	175
PDE3B	phosphodiesterase 3B	126
PDH	pyruvate dehydrogenase complex	35
PDK4	pyruvate dehydrogenase kinase 4	147
$PETCO_2$	partial pressure of end tidal carbon dioxide	75
PFK	6-phosphofructo-1-kinase	35
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator 1-alpha	47
P_i	inorganic phosphate	23
PLIN1	perilipin 1	126
PPIA	peptidylprolyl isomerase A	64
Q	ubiquinone	33
QH_2	ubiquinol	33
qPCR	quantitative polymerase chain reaction	63
Ra	rate of appearance	106

R_d	rate of disappearance	45
RER	respiratory exchange ratio	12
RM	repeated measures	71
RNA	ribonucleic acid	63
RPE	ratings of perceived exertion	67
RPM	revolutions per min	52
SCOT	succinyl-CoA: 3-ketoacid CoA transferase	28
SDS	sodium dodecyl sulfate	60
SvO2	mixed venous oxygen saturation	175
TCA	tricarboxylic acid	22
tris	tris(hydroxymethyl)aminomethane	60
TTR	tracer to tracee ratio	93
T_{Vent}	ventilatory threshold	43
VCO_2	volume of carbon dioxide expelled	53
V_E	ventilatory exchange	53
VO_2	volume of oxygen consumed	52
$VO_{2\ Max}$	maximum oxygen uptake	24
$VO_{2\ peak}$	peak oxygen uptake	25
WADA	World Anti-Doping Authority	51
W_{Max}	maximal power	1

Declaration

I declare that this thesis has been composed solely by myself and that it has not been submitted, in whole or in part, in any previous application for a degree. Except where acknowledged below, the work presented is entirely my own.

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- Dearlove D. J., Faull O. K., Clarke K. Context is key: exogenous ketosis and athletic performance. In: *Curr Opin Physiol* 10 (2019), pp. 81-89 (invited review paper)

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Chapter 1

Introduction

It has long been recognised that the content of our diet may influence physical performance [1]. Accordingly, in pursuit of making athletes "faster, higher and stronger" [2], a large volume of research has been undertaken seeking to determine "optimal" dietary strategies. This work has predominately focused on manipulating the intake of dietary macronutrients: CHO, fat and protein. Work presented in this Thesis examines the effects on exercise physiology, metabolism and capacity of supplementing a "fourth" major macronutrient: the ketone body, βHB. To understand the biochemical rationale for supplementing athletes with ketones, limitations of substrate metabolism during exercise must first be introduced.

1.1 Substrate metabolism during exercise

1.1.1 Interaction between energy systems

Muscular contraction is reliant upon the hydrolysis of adenosine triphosphate (ATP) and subsequent release of free energy. Given its importance, the total store of ATP within the cells is surprisingly small ($\approx$8 mM·kg wet weight^{-1}) [3]. As such, a perpetual replenishment of the intramuscular ATP is required for continued muscular contraction. This requirement is particularly pronounced during high-intensity exercise, where ATP turnover may increase 1,000-fold above baseline [4]. To meet the requirement for rapid ATP synthesis, cells rely a complex set of catabolic pathways, predominantly: the phosphagen system, the glycolytic system and mitochondrial

phosphorylation. Collectively, these pathways are remarkably effective at maintaining cellular ATP homeostasis. Indeed, even in instances of involuntary maximal muscular contraction to failure, muscle ATP is not lower than ≈5 mM·kg wet weight^{-1} [5].

1.1.2 Phosphagen system

Creatine phosphate is stored in the cytosol, in close proximity to sites of energy utilisation. The phosphocreatine system consists of three reactions (1.1), meaning ATP resynthesis is rapid.

$$\begin{aligned} CrP + ADP + H^+ &\xrightarrow{creatine\ kinase} ATP + creatine \\ ADP + ADP &\xrightarrow{adenylate\ kinase} ATP + AMP \\ AMP + H^+ &\xrightarrow{AMP\ deaminase} IMP + NH4^+ \end{aligned} \tag{1.1}$$

As a consequence, it supports maximal intensity exercise lasting ≈5 - 6 sec [6], which requires very high rates ATP resynthesis. However, creatine phosphate (CrP) stores are largely depleted within ≈10 sec of maximal exercise [7].

1.1.3 Glycolytic system

Almost immediately upon commencement of exercise, a rapid increase in the production of glucose 6 phosphate (G6P) from glycogenolysis and blood glucose uptake occurs [3]. Through a series of catabolic reactions termed glycolysis (overall reaction presented in equation 1.2), these substrates produce two molecules of pyruvate, a net gain of two ATP (three ATP when G6P is the starting substrate [3]) and two molecules of reduced nicotinamide adenine dinucleotide (NADH). Glycolysis contributes to ATP resynthesis almost instantaneously during maximal exertion exercise [8, 9], achieving a maximal rate after ≈10 - 15 secs. The catabolism of G6P and glucose requires more reactions versus the phosphagen system, making ATP

resynthesis comparatively slower [10]. For maximal exercise bouts lasting ≈30 sec - 3 min, glycolysis is the predominant energy system [11]. Glycolysis results in a net increase in cytosolic proton (H^+), resulting in a metabolic acidosis [12].

$$\begin{aligned} &glucose + 2\ NAD^+ + 2\ ADP + 2\ P_i \longrightarrow \\ &2\ pyruvate + 2\ NADH + 2\ H^+ + 2\ ATP + 2\ H_2O \end{aligned} \tag{1.2}$$

Glycolysis represents only partial oxidation of G6P or glucose. Pyruvate may be decarboxylated to form acetyl-coenzyme A (Ac-CoA), which may then enter the tricarboxylic acid (TCA) cycle for complete oxidation (discussed below). However, during high-intensity exercise, glycolytic flux results in a production of pyruvate that exceeds the capacity of mitochondrial phosphorylation. "Excess" pyruvate is diverted towards the production of lactate via the lactate dehydrogenase reaction. The mechanism for this diversion has classically been attributed to a depletion of intracellular oxygen [13]; however, this is misleading [12]. Whilst a reduction in oxygen availability may contribute, exercise of sufficient intensity will always result in lactate production, regardless of normal oxygenation [12].

The lactate dehydrogenase reaction has several advantages that help to sustain continued muscular contraction. Firstly, the formation of lactate prevents product inhibition of pyruvate on glycolysis [14]. Secondly, this reaction replenishes the cytosolic oxidised nicotinamide adenine dinucleotide (NAD^+) pool, which is required for the conversion of glyceraldehyde 3-phosphate (G3P) to 1,3-bisphosphoglycerate (1,3-BPG) in glycolysis [15] and, therefore, facilitates continued glycolytic flux. Furthermore, the lactate dehydrogenase reaction consumes a free H^+ and thus retards the metabolic acidosis associated with high-intensity exercise [12]. Finally, lactate may be shuttled between tissues and may provide a quantitatively important substrate for the TCA cycle and, therefore, ATP synthesis [16].

1.1.4 Mitochondrial phosphorylation

The mitochondrial phosphorylation of substrates yields significantly more ATP versus phosphagen and glycolytic systems [17]. However, it is relatively laboured and requires sufficient oxygenation [10], thus it predominates during longer-duration, relatively low-intensity exercise. Unlike the pathways described previously, mitochondrial phosphorylation is not fixed to a single substrate. During exercise, fuel for ATP resynthesis by mitochondrial phosphorylation is predominately derived from intracellular stores of fat (in the form of triacylglycerol molecules) and CHO (in the form of glycogen), and to a lesser extent circulating free fatty acid (FFA) and glucose [18]. Given that protein has a functional purpose within the cell, skeletal muscle catabolism does not significantly contribute to energy expenditure in normal metabolic settings [19, 20].

As alluded to above, pyruvate and the NADH derived from glycolysis may enter the mitochondria for oxidative phosphorylation (frequently referred to as aerobic glycolysis). Medium- and long-chain fatty acids, predominately palmitic acid [21], are activated to fatty acyl-coenzyme As (acyl-CoAs) prior to facilitated transport into the mitochondria via the carnitine shuttle. Once within the mitochondria, fatty acyls are reduced two carbons at a time through the β-oxidation pathway, yielding one Ac-CoA, NADH and oxidised flavin adenine dinucleotide ($FADH_2$) per cycle (the overall reaction for one cycle of β-oxidation is presented in equation 1.3). Thus, glucose and fat oxidation converge at the point of Ac-CoA incorporation into the TCA cycle. As such, differences in the number of ATP synthesised through oxidation of glucose and fat occur prior to the production of Ac-CoA (discussed later). Oxidative ATP synthesis is complete when electrons and protons derived from the reduction of NADH and $FADH_2$ are pumped across the inner mitochondrial membrane creating an electrochemical gradient that drives the F_1F_0ATPase complex towards the formation of ATP from adenosine diphosphate (ADP) + inorganic phosphate (P_i).

$$C_n\text{-}acyl\text{-}CoA + FAD^+ + NAD^+ + H_2O + CoA \rightarrow$$
$$C_{n\text{-}2}\text{-}acyl\text{-}CoA + FADH_2 + NADH + H^+ + acetyl\text{-}CoA \quad (1.3)$$

1.1.5 Moderate- to high-intensity exercise is largely reliant on intramuscular glycogen catabolism

The relative contribution of energy systems and substrates to ATP synthesis is heavily influenced by the intensity and duration of exercise [18]. For reasons described above, the mitochondrial oxidation of fat and CHO fulfills most of skeletal muscles' energy requirements during endurance exercise, for which this Thesis is predominately concerned. The relationship between fat oxidation and exercise intensity is typically parabolic. Fat oxidation rates increase from low to moderate intensities, before declining at higher workloads ($\approx \geq$75% maximum oxygen uptake ($VO_{2\ Max}$)) [22]. The mechanisms governing the suppression of fat oxidation during high-intensity exercise include: decreased transport of circulating FFA into the myocyte [23]; reduced binding of FFA to the fatty acid binding protein and subsequent impaired intracellular transport [24]; decreased intramuscular triglyceride breakdown [25]; and reduced transport of long-chain fatty acids into the mitochondria due to decreased free carnitine and/or carnitine palmitoyltransferase I (CPT I) activity [26].

Conversely, CHO oxidation increases proportionally to exercise intensity, with exercise $\geq$75% $VO_{2\ Max}$ being largely reliant on the catabolism of intramuscular glycogen stores [18] (see Figure 1.1, which illustrates the relative contributions of fat and CHO to overall energy expenditure during incremental intensity cycling in a highly trained endurance athlete). Given that the rate of ATP resynthesis from the oxidation of fat is two to three times slower (0.24 mmol·sec^{-1}·kg BW^{-1}) than CHO (0.51 - 0.68 mmol·sec^{-1}·kg BW^{-1}) [27] and that the ratio of ATP synthesised to oxygen consumed (P/O ratio) is lower for CHO [26], the reliance on glycogen catabolism

at high exercise intensities is logical. However, glycogen stores are finite, representing ≈500 g [28]. Consequently, glycogen availability is an important determinant of physical capacity, both in prolonged (≥1 hour in duration) and high-intensity intermittent exercise [29–35].

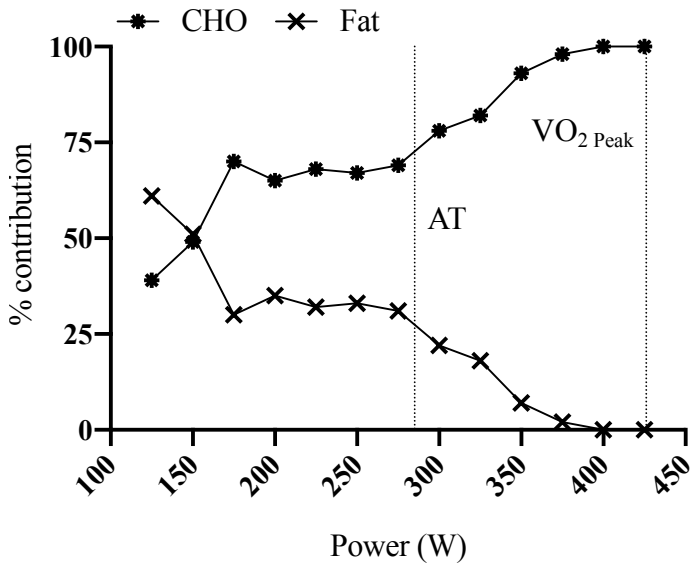


Figure 1.1: **Interaction between substrate oxidation and exercise duration**. Data presented here is taken from a cycling ergometer incremental intensity test (25 W increments every 3 min) performed on a highly-trained endurance athlete. CHO rapidly becomes the major energy substrate during incremental intensity exercise, with the switching point occurring in this athlete at ≈35% peak oxygen uptake ($VO_{2\ peak}$). Reliance on CHO increases sequentially at exercise intensities above the anaerobic threshold (indicated by the first vertical dotted line), where CHO utilisation was ≈120 $g{\cdot}h^{-1}$. Importantly, success in many endurance sports requires sustaining exercise at these intensities for prolonged periods and thus, is highly associated with intramuscular glycogen availability [33].

1.1.6 Effect of endurance exercise training on mitochondrial fuel selection

Endurance exercise training causes a "remodeling" of skeletal muscle [36]. This includes changes in mitochondrial function [37] and volume [38], and enzymes that regulate metabolism [39] and intracellular signalling [40]. The net effect of these adaptations is to enhance fatigue resistance for future exercise bouts [36]. An important metabolic manifestation of muscular remodeling is increased FFA oxidation

during sub-maximal exercise intensities [41, 42], thus highlighting the importance of sparing finite glycogen stores for fatigue resistance.

1.1.7 Summary

In sum, muscular contraction is primarily reliant on transforming energy stored in both simple molecules (as in creatine phosphate) and complex macromolecules (as in glycogen and triacylglycerol) into mechanical energy through the synthesis of ATP. Intramuscular stores of ATP are surprisingly small. As such, a complex and highly regulated set of metabolic pathways exist to ensure the ATP pool is not depleted. The size and availability of energy depots (both within the muscle and from the circulation), and the speed in which they can be metabolised for the synthesis of ATP largely dictates the relative contribution of energy systems. Competitive endurance exercise typically requires sustaining an intensity at which intramuscular glycogen catabolism and CHO oxidation rates are high. As such, glycogen availability is a strong determinant of performance. Given this, exercise physiologists, trainers and athletes are motivated to find methods to either: (i) restrict the reliance on glycogen catabolism at high exercise intensities; or (ii) increase the size of glycogen stores. In pursuit of these goals, most work has focused on altering the types, timings and amount of fat and CHO ingested with varying degrees of success [43–46].

1.2 Ketosis: a metabolic adaptation to energy crisis

In states of decreased CHO availability such as starvation, circulating ketone bodies - mainly βHB and acetoacetate (AcAc) - are elevated and represent an additional major energy substrate [47, 48]. However, ketones are more than a simple oxidisable carbon source [49]; rather, they have an array of signalling functions that collectively act to maintain energy homeostasis by altering whole body substrate metabolism [48, 49]. As will now be discussed, ketone metabolism and signalling - pivotal

to survival during starvation [19, 47] - might also be utilised to enhance human endurance exercise capacity.

1.2.1 Overview of endogenous ketone metabolism

Blood ketone concentrations rise rapidly in response to physiological and pathological states characterised by elevated circulating FFA concentrations (Table 1.1). Ketogenesis occurs primarily within the mitochondrial matrix of hepatocytes and has traditionally been viewed as a β-oxidation "spillover" pathway [50] (Figure 1.2). When the rate of Ac-CoA formation derived from the β-oxidation of fatty-acyls exceeds citrate synthase (CS) activity and/or oxaloacetate availability (required for its condensation to citrate for subsequent entry into the TCA cycle), "excess" Ac-CoA is condensed to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) in the fate committing (irreversible) reaction of ketogenesis [51]. Hydroxymethylglutaryl-CoA lyase (HMGCL) cleaves HMG-CoA to form Ac-CoA and AcAc, the latter of which may subsequently be reduced to form βHB. The mechanisms by which ketones move across mitochondrial membranes have not been fully elucidated but are thought to occur through the mitochondrial pyruvate carrier (MPC) and/or monocarboxylic acid transporter (MCT)1/2 [52]. AcAc and βHB are transported across the hepatocyte cell membrane by MCT1/2 [52] and enter the circulation primarily for uptake and metabolic disposal in extrahepatic tissues [50]. βHB is the predominant ketone in the circulation, with concentrations being 3- to 5-fold greater than AcAc [48].

The primary fate of ketones is terminal oxidation within extrahepatic tissues [50] in a process termed ketolysis (overall reaction presented in equation 1.4).

$$\begin{aligned} &\beta HB + NAD^{+} + \textit{succinyl-CoA} + CoA \rightarrow \\ &NADH + H^{+} + \textit{succinate} + \textit{2 acetyl-CoA} \end{aligned} \tag{1.4}$$

Within the mitochondria of extrahepatic tissues, βHB is oxidised to form AcAc in

Table 1.1: **Approximate range of human blood ketone concentration in response to ketogenic stimuli.** Values represent the combined concentration of βHB and AcAc measured in whole blood (typical ratio of 3:1 to 5:1) [47, 48].

	Approx. blood ketones (mM)
Post CHO containing meal	$\leq$0.1
Overnight fast	0.2 - 0.3
Post exercise	1 - 2
3-day fast	2 - 3
Prolonged ketogenic diet	3 - 5
1-week fast	5 - 6
4-to-5-week starvation	Up to 7.5
Diabetic ketoacidaemia	Up to 25

a NAD^+/NADH near-equilibrium reaction (Figure 1.2). The rate-limiting reaction of ketolysis is the conversion of AcAc to acetoacetyl-CoA (AcAc-CoA), catalysed by succinyl-CoA: 3-ketoacid CoA transferase (SCOT). Finally, AcAc-CoA is converted to two molecules of Ac-CoA, which may then enter the TCA cycle. AcAc may "escape" oxidation through mitochondrial export, where it may be used as substrate for *de novo* lipogenesis (DNL) and cholesterogenesis [48, 50]. Furthermore, ketones may be excreted from the body within the urine [53] or, following the spontaneous decarboxylation of AcAc, as acetone in the breath [54] (observed as the "sweet" breath smell in ketotic individuals).

1.2.2 Sites of ketone utilisation

Brain

An apparent peculiarity of evolution is that the brain does not meaningfully utilise our largest energy store, fat. The evolutionary rationale for this has not been fully elucidated but may include protecting neurons from the oxidative stress associated with fat oxidation and the relatively slow pace of ATP resynthesis via the combustion of fat versus carbohydrate [27]. Rather, in normal metabolic settings it is

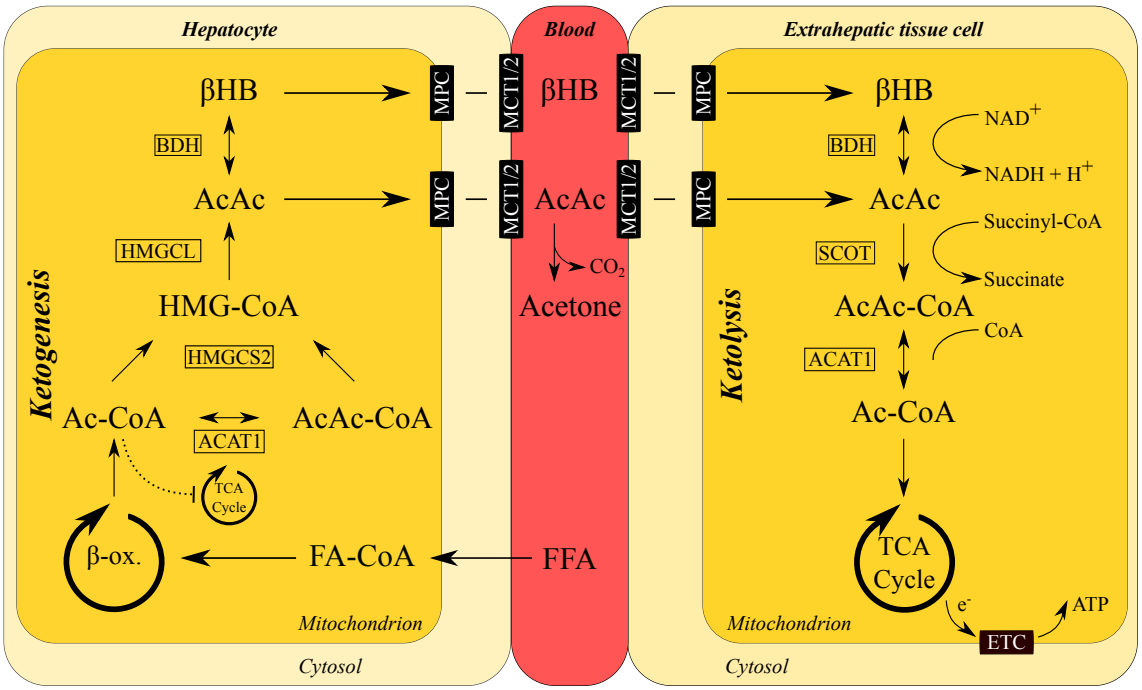


Figure 1.2: **Overview of ketone metabolism. Ketogenesis (left)**: Hepatic ketogenesis is stimulated by an increase in blood FFA concentration. Ac-CoA that exceeds TCA cycle capacity is directed towards ketone synthesis. **Ketolysis (right)**: The primary metabolic fate of ketones is terminal oxidation in extrahepatic tissues. Abbreviations not previously defined: ETC = electron transport chain, acetyl-CoA acetyltransferase 1 (ACAT1).

almost entirely reliant on CHO, which constitutes ≤1% of total energy stored within the body [47]. Conversely, lipid-derived ketones are avidly consumed by the brain and nerve tissue [55]; thus, ketogenesis provides a mechanism to link energy stored in fat to cerebral metabolism when dietary CHO intake is restricted [47, 55]. Indeed, seminal work by Owen and colleagues [55] demonstrated that ketones account for ≈60% of cerebral oxygen consumption following five to six weeks of starvation. Ketogenesis is observed in all higher order organisms, but its importance is particularly pronounced in homo sapiens due to a comparatively large brain and its associated high energy requirements (representing ≈20% of energy requirements at rest) [56]. Whilst the primarily purpose of ketogenesis is to provide a supplementary fuel (to glucose) for cerebral metabolism, most extrahepatic tissues may utilise ketones, including skeletal muscle [48, 57]. The mechanisms governing the preferential utilisation of ketones between body tissues have not yet been elucidated.

Muscle

Cardiac tissue has the highest rate of ketone utilisation [48]. Indeed, ketones appear to be preferentially oxidised by cardiac muscle (versus fat and CHO), having been shown to account for 83% of carbon dioxide (CO_2) production in the perfused canine heart [58]. In contrast, ketone utilisation is substantially lower in skeletal muscle (approximately one tenth of that of the heart [59]). In part, this disparity may be explained by the large differences in ketolytic enzyme activity between these tissues [48, 60] (see enzyme activity rates, Table 1.2).

Whilst per gram of tissue skeletal muscle has a comparatively low ketolytic capacity [48, 60], it represents ≈40% of total body mass [61] (see percentage body mass, Table 1.2; although large variability in the relative proportion of skeletal muscle mass to overall body mass is present in the population [62]) and as such, is the largest potential site for ketone uptake and utilisation. Furthermore, unlike the heart, skeletal muscle is not continually working. This is reflected by its relatively low energy demands at rest [63]. During vigorous exercise, however, a substantial proportion of skeletal muscle mass may be activated, with a concomitant ≥1,000-fold increase in ATP synthesis [3] and 4-fold increase in cardiac output to this tissue [64] (see percentage cardiac output, Table 1.2). Thus, during exercise, skeletal muscle is the main site of substrate metabolism [36]. Taken together, this suggests that *active* skeletal muscle may be a potentially important site of ketone utilisation.

To date, the strongest evidence for the importance of ketone oxidation in muscle comes from Balasse and Féry [57], who demonstrated that the metabolic clearance rate of ketones (an index of the capacity of working skeletal muscle to extract ketones from the blood) is increased ≈40% upon commencement of exercise in overnight fasted subjects (blood ketone concentration of ≈0.2 mM). This increase is similar to that observed for FFA and CHO [57] and is consistent with ketones being a respiratory fuel in skeletal muscle. Of course, given the low blood concentrations, ketones would not meaningfully contribute to overall energy expenditure following an overnight fast. Notably, Balasse and Féry [57] observed that the stimulative

Table 1.2: **Sites of ketone utilisation.** SCOT and β-hydroxybutyrate dehydrogenase (BDH) enzyme activity relate to rat tissue [48]. Estimations of the relative contribution of tissues to overall body mass refer to males only [61]. Estimations of percentage distribution of cardiac output to body tissues at rest and during heavy exercise were taken from Frayn and Evans [64]. * = unknown.

	Enzyme activity (μM.min^{-1})				
	BDH	**SCOT**	**% body mass**	**% CO (rest)**	**% CO (exercise)**
Adipose tissue	*	0.3	11.4%	5 - 10%	1%
Brain	1.5	2.1	3.0%	15%	3 - 4%
Heart	2.5	26	0.5%	4 - 5%	4 - 5%
Kidney	4	26	0.5%	20%	2 - 4%
Lung	*	1.6	3.3%	100%	100%
Skeletal muscle	0.6	0.8	39.8%	15 - 20%	80 - 85%

effect of exercise was less pronounced after a two-to-three-day fast (blood ketone concentration of ≈3 mM) and abolished after a three-to-five-day fast (blood ketone concentration of ≈5.5 mM) suggesting ketone uptake in skeletal muscle is saturated at relatively low circulating concentrations. This contrasts directly to the brain, where uptake is proportional to arterial concentrations [65], an effect that is mediated by greater permeability across the blood-brain barrier, rather than increased ketolytic enzyme activity [66–68]. This observation likely reflects the greater importance of ketone oxidation in this tissue, which unlike skeletal muscle cannot meet its energy requirements from fat oxidation [36].

1.2.3 Ketones may be energy efficient substrates

The oxidation of ketones may have thermodynamic advantages versus other substrates [17, 69, 70]. As previously discussed, an electrochemical gradient that is created through the pumping of H^+ across the inner mitochondrial membrane drives the F_1F_0ATPase complex towards the formation of ATP from ADP and P_i. The gradient generated is determined by differences in electrochemical potential of

the NAD^+/NADH couple and co-enzyme Q couple. βHB (the predominant ketone body) is more chemically reduced than glucose, pyruvate and lactate [17, 70] (see Figure 1.3) resulting in a reduction (decrease) of the NAD^+/NADH ratio, whilst simultaneously oxidising (increasing) the co-enzyme Q couple [17, 69]. By increasing the redox span, when electrons are passed from NADH to co-enzyme Q a greater number of protons can be pumped across the inner mitochondrial membrane. This subsequently increases the electrochemical gradient, thereby increasing ATP synthesis [17, 69]. This may be conceptualised by thinking of the NAD^+/NADH couple and co-enzyme Q couples as magnets. βHB oxidation increases the magnetic "pull" towards co-enzyme Q, therefore increasing the number of electrons passed down the chain and, subsequently, the number of protons pumped across the inner mitochondrial membrane. This effect may explain the 25% greater hydraulic efficiency ($J \cdot mol^{-1}$ of oxygen (O_2) consumed) in the perfused, working rat heart when a physiological ratio of ketones was added to a 10 mM glucose buffer, versus glucose alone [69]. This study was performed in hearts perfused with 10 mM glucose to which 100 mM of insulin or 4 mM of βHB and 1 mM AcAc were added. The exclusion of fatty acids from the perfusate likely reduced the physiological relevance of these findings.

Whilst fat is highly reduced (Table 1.3), its contribution to energy production is restricted when ATP must be resynthesised rapidly [18, 26] (see section 1.1.5). Conversely, the effect of the degree of physical exertion on βHB oxidation rates is currently unclear, although blood βHB clearance is increased at higher exercise intensities [71] implying increased oxidation. This limits the utility of fat to power muscular contraction during high-intensity exercise [3, 26]. Furthermore, due to half of the electrons being donated to the electron transport chain via $FADH_2$, there is a greater "oxygen cost" [17] for fat versus ketones. Additionally, elevated FFA concentrations induce the expression of uncoupling proteins, which dissipate the electrochemical gradient across the inner mitochondrial membrane [72], thus further reducing efficiency.

Potential improvements in energy efficiency from ketone metabolism (that is, the

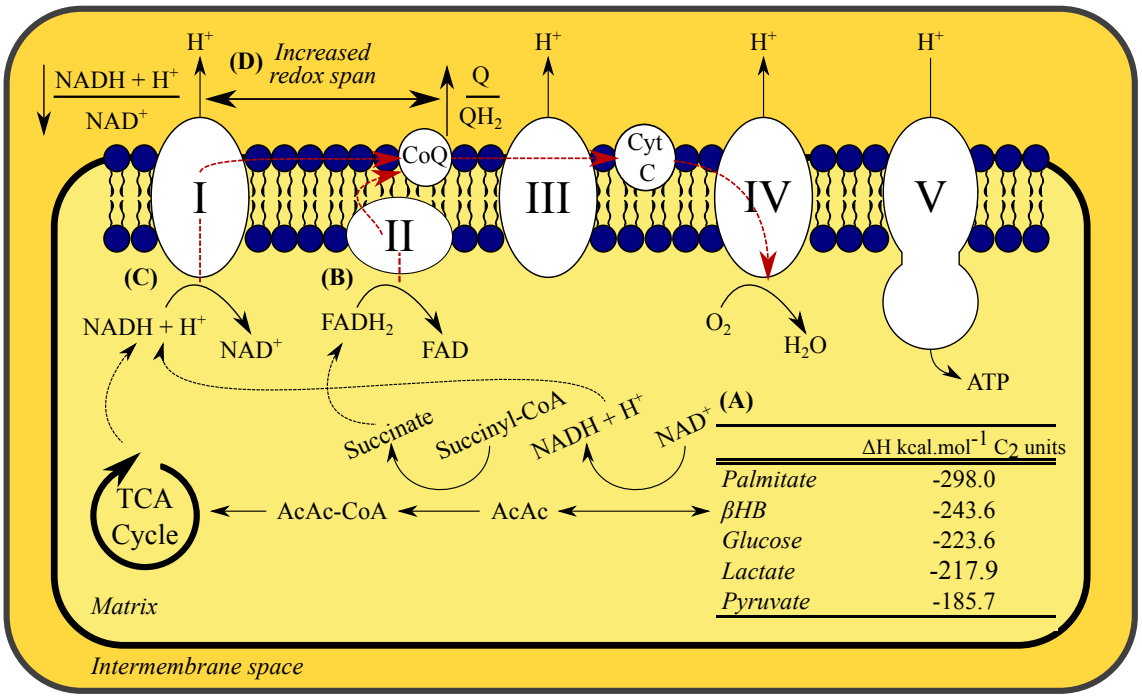


Figure 1.3: **Effect of βHB metabolism on mitochondrial energetics. (A)** Per mole of C_2, βHB is more chemically reduced versus CHO derived substrates. **(B)** The conversion of AcAc to AcAc-CoA yields succinate. This may subsequently be converted to fumarate and $FADH_2$ in a reaction catalysed by succinate dehydrogenase. $FADH_2$ may then provide electrons to complex II of the electron transport chain. **(C)** NADH + H^+ is produced from the dehydrogenation of βHB to AcAc and from Ac-CoA catabolism within the TCA cycle. NADH donates electrons at complex I. **(D)** βHB oxidation decreases the NAD^+/NADH and increases the ubiquinone (Q)/ubiquinol (QH_2) couples, thus increasing the redox span. More energy can then be liberated from the transfer of electrons from NADH to Q, resulting in increased ATP synthesis.

capacity to expend less energy for a given amount of work [73] or level of oxygen consumption [70]) have clear advantages to survival during starvation [19]. If ketone metabolism could be effectively harnessed outside the context of starvation, it may also provide a method to enhance human physical performance.

1.2.4 Ketone signalling

In addition to providing an additional substrate, ketones have an array of metabolic signalling actions that regulate the availability and catabolism of other fuels [49].

Decreased glucose utilisation

It has been recognised for almost a century that ketones inhibit glycolysis [74], thus protecting finite CHO stores during starvation [19, 47]. This effect is apparent in the heart [75, 76], muscle [77, 78] and brain [79]. Given that depleting intramuscular glycogen is strongly associated with exercise fatigue [33–35], the ketone-mediated suppression of glycolysis may, in some contexts [80], provide a method to enhance human physical capacity. Randle and colleagues proposed a model of substrate level inhibition to explain the effect (Randle cycle [14]), which is depicted in steps 1 - 4 of Figure 1.4.

Reduced muscle protein catabolism, hypoalaninaemia and hypoglycaemia

Both physiological [81] and pathological [82] ketoses are accompanied by reductions in blood alanine concentrations. Hypoalaninaemia is believed to result from decreased muscle output [83] and has been linked to the ketone-mediated suppression of glycolysis (see steps 1 - 3 and 6, Figure 1.4) [83, 84]. Alternatively, ketones may inhibit alanine release through a direct reduction in muscle protein catabolism (step 5, Figure 1.4). This mechanism is supported by the decrease in urinary urea observed following a prolonged fast [47] and the observation that whole-body alanine and leucine oxidation is reduced following intravenous infusion of ketones in overnight fasted subjects [83, 85–87]. The decrease in muscle protein catabolism observed during the transition from brief to prolonged fasting is a pivotal metabolic adaption that helps to sustain life during starvation [19].

The ketone-mediated suppression of glycolysis, muscle protein catabolism and muscle protein synthesis may also provide an indirect method to regulate hepatic gluconeogenesis during starvation by limiting the supply of alanine - an important gluconeogenic substrate [88] - to the liver. This may explain the significant reductions in blood glucose concentrations in overnight fasted individuals (where hepatic gluconeogenesis is a significant contributor to glycaemia [89]) infused or supplemented with ketones [90–92].

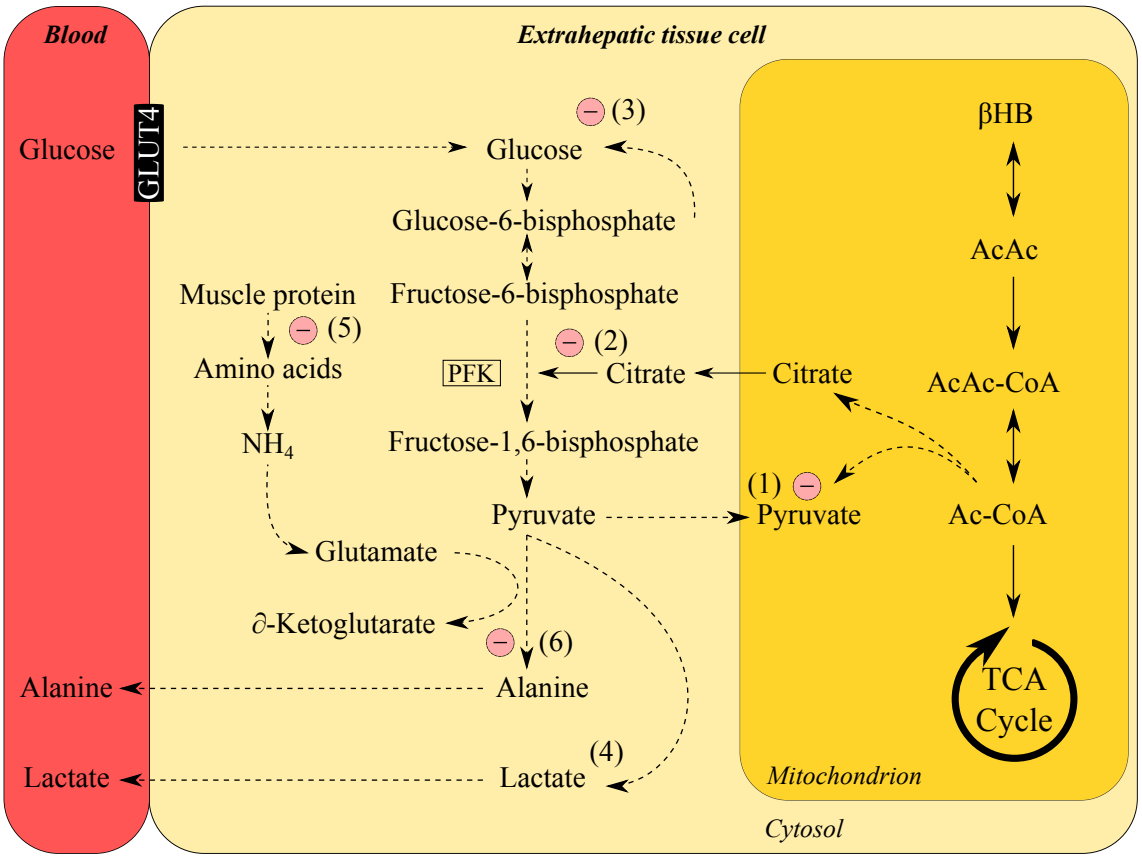


Figure 1.4: **Proposed mechanisms for the inhibition of glycolysis and hypoalaninaemia**. **(1)** An accumulation of mitochondrial Ac-CoA from ketone oxidation may cause product inhibition on the pyruvate dehydrogenase complex (PDH) reaction, thus preventing pyruvate formation. **(2)** Ac-CoA formed from ketone oxidation and not incorporated into the TCA cycle is converted to citrate and exported into the cytosol where it may inhibit 6-phosphofructo-1-kinase (PFK). **(3)** This results in an accumulation of the "upstream" metabolite, G6P, which subsequently prevents glucose uptake by product inhibition of hexokinase. **(4)** Reductions in glycolysis during ketosis may cause a reduced flux through the lactate dehydrogenase (LDH) reaction, leading to lower intra- and extra-cellular lactate concentrations. **(5)** Muscle protein catabolism is decreased during starvation, as observed by significant reductions in urinary urea [47]. **(6)** The suppression of glycolysis reduces the availability of pyruvate for transamination with amino acids to form alanine. Dotted lines represent down-regulated pathways. Abbreviations not previously defined: ammonium (NH_4).

Reduction in blood free fatty acid concentrations

βHB and AcAc have antilipolytic effects, mediated through antagonism of the hydroxycarboxylic acid receptor 2 (HCAR2) receptor on adipocyte cell membranes [93]. This is considered to be a negative feedback loop that restricts the supply of FFA to the liver (substrate for hepatic ketogenesis), thus preventing circulating

ketone concentrations exceeding ≈7 mM [48] and the subsequent induction of ketoacidaemia (observed in pathological ketotic states such as uncontrolled diabetes) [48].

Reduction in sympathetic tone

βHB exerts an antagonistic effect on the free fatty acid receptor 3 (FFAR3), which is highly expressed throughout the peripheral nervous system in mice [94]. FFAR3 is activated by short chain fatty acids, including proprionate, resulting in increased sympathetic nervous stimulation that manifests as high energy expenditure and heart rate (HR) in mice [95]. βHB suppresses propionate-induced sympathetic activation in both *ex vivo* and *in vivo* models [96]. As sympathetic nervous activity is associated with catabolic or energy expending processes [97], in the context of a starvation ketosis it makes teleological sense that βHB would act as an antagonist for FFAR3.

1.2.5 Exercise during an endogenously-induced ketosis

Driven by the aforementioned limitations of CHO metabolism (that is, intramuscular glycogen being a finite fuel store), there is sustained interest in the use of very low CHO diets (typically ≤10% of daily energy intake from CHO [98]) to enhance exercise performance [99–102]. Low blood insulin and elevated FFA concentrations mimic the metabolic conditions of starvation and thus stimulate hepatic ketogenesis (hence them being interchangeably referred to as ketogenic diets).

Importantly, the proposed metabolic advantages of exercising during a ketogenic diet relate to increased fat oxidation, rather than ketone oxidation or signalling [99]. Briefly, a prolonged ketogenic diet causes metabolic adaptations that enhance the capacity for fat oxidation in skeletal muscle [103]. Indeed, adoption of a chronic ketogenic diet (≥1 year) may yield an ≈2-fold increase in maximum fat oxidation rate [103]. However, concomitant reductions in the capacity for CHO oxidation are observed, mediated by a reduction in intramuscular glycogen content (at least

in the short-term) [104] and decreased glycolytic enzyme activity [105]. This loss of skeletal muscle metabolic flexibility may limit performance during high-intensity exercise [43]. Thus, whilst ketone metabolism may advantageously effect exercise metabolism and performance, this is unlikely to be realised during an endogenously induced ketosis (that is, through starvation or ketogenic diet).

1.2.6 Summary

In sum, ketosis acts to maintain energy homeostasis during starvation by providing an alternative and energy-efficient fuel for extrahepatic tissues, whilst simultaneously altering availability and catabolism of other substrates [47, 48]. There are clear parallels between the metabolic demands of endurance exercise (see paragraph 1.1.7 for a summary) and starvation. In both states, preserving finite CHO stores and maximising energy efficiency (the capacity to expend less energy for a given workload) is a priority [26, 33, 47]. As such, ketone metabolism, which is vital to maintaining energy homeostasis during starvation, may also provide a method to enhance human endurance exercise capacity [70]. However, due to a loss of metabolic flexibility resulting from a reduction in glycogen stores and glycolytic enzyme activity, the potentially advantageous effects of ketosis on human metabolism are unlikely to translate to increased exercise performance during an endogenously-induced ketosis [43, 70, 102].

1.3 Exogenous ketosis: a novel metabolic state

The ingestion of ketones creates a novel metabolic state, "exogenous ketosis", which may allow us to harness the potentially advantageous aspects of ketone metabolism [70, 106] without the loss of skeletal muscle metabolic flexibility observed with a ketogenic diet [43, 101, 102, 104, 105] (see Table 1.3).

Table 1.3: **Comparison of endogenously- and exogenously-induced ketoses** [19, 71, 104, 107, 108].

	[Insulin]	[FFA]	[Glucose]	[Alanine]	[IMGLY]
Starvation	↓	↑	↓	↓	↓
Ketogenic diet	↓	↑	↓	↓	↓
Exogenous ketosis	- / ↑	↓	↓	↓	- / ↑

1.3.1 The chemical composition of exogenous ketones may determine their effectiveness

Oral ketone supplements may be delivered as salts or esters [92]. The effectiveness of these supplements largely depends on their chemical composition [92, 109]. Ketone salt supplements are comparatively ineffective at elevating circulating ketone concentrations [110–112]. This is likely due to most salt supplements providing racemic mixtures of the bioactive D-βHB isoform and the less characterised L-βHB isoform [92], which is synthesised in extrahepatic tissues from AcAc-CoA when it accumulates due to a TCA cycle bottleneck [50]. Only the D-βHB isoform is converted into AcAc for mitochondrial oxidation and as such, elevating L-βHB concentrations is unlikely to affect exercise substrate metabolism [110–112]. Additionally, the amount of sodium delivered through ketone salt consumption may be deleterious to health [113], so prolonged supplementation is undesirable.

Two ketone esters have been trialed and have been shown to safely and rapidly elevate blood ketone concentrations [114, 115]. These compounds are made up of one or more ketone molecules (AcAc or βHB) joined with an ester bond to a ketone precursor, such as butanediol. Once consumed, the ester bonds are hydrolysed by ubiquitously expressed gut esterases and the ketones released into the blood stream. Butanediol is processed by the liver to form further βHB and AcAc molecules. It is possible to achieve blood ketone concentrations approaching the physiological limit of $\approx$7 mM in a dose-dependent fashion [115, 116], which may remain elevated for several hours after ingestion [92].

There are a number of biochemical advantages of using a βHB versus AcAc ester. Firstly, the MPC (which is believed to facilitate the transport of βHB and AcAc into the mitochondria [117, 118]) has a very high affinity for AcAc (0.61 mM) compared to βHB (5.6 mM) [118]. This likely reflects the ≈4-fold higher plasma concentration of βHB during a starvation- or dietary-induced ketosis [48]. Consuming large quantities of exogenous AcAc and thus perturbing the normal AcAc:βHB ratio may saturate the MPC (that is, the enzyme will work at a constant rate, regardless of increased intracellular AcAc), potentially causing a bottleneck for its oxidation. Secondly, βHB is more chemically reduced than AcAc. As previously described, the relative supply of reducing equivalents to the mitochondrial electron transport chain alters the proton motive force and, therefore, energy available for ATP synthesis. Thus, the potential energy transduction benefits achieved through an exogenously-induced ketosis could be blunted when using an AcAc versus βHB ester. Finally, whilst the βHB ester has an unpleasant taste, it is generally well tolerated [119]. Conversely, the only study to test the effects of the AcAc ester on human athletic performance found that all athletes suffered moderate to severe gastrointestinal distress, which very likely hampered exercise performance [120]. For these reasons, all subsequent discussions regarding the effects of an exogenously-induced ketosis will relate to the βHB monoester (herein referred to as ketone monoester).

1.3.2 Effects of an exogenously-induced ketosis on substrate metabolism during exercise

A limited number of studies have reported the effects of an exogenously-induced ketosis on substrate metabolism during acute exercise [71, 112, 121–123]. These effects are summarised in Table 1.4 and discussed below.

Respiratory fuel

Just one study has sought to investigate the metabolic disposal of βHB in exercise following ketone monoester ingestion. Cox and colleagues [71] predicted that

the oxygen consumption attributable to βHB oxidation was 18% and 16% at workloads of 40% and 75% W_{Max}, respectively [71]. This equated to a βHB oxidation rate of ≈0.3 - 0.5 g·min^{-1}, similar to rates reported for FFAs and other fat sources [18]. However, in lieu of direct measures of βHB oxidation, this work relied on adjusted substrate oxidation equations (conventional equations being confounded by the presence of a third potentially major substrate) using gaseous exchanges [20]. As such, further work is warranted to determine the contribution of exogenous βHB oxidation to overall energy expenditure in exercising skeletal muscle using direct measurement techniques. Furthermore, the factors regulating βHB oxidation rates, including blood βHB concentrations and the intensity of exercise performed, have not been determined.

Signalling molecule

As discussed in paragraph 1.2.4, ketones have pleiotropic effects on metabolism, including acting as signalling molecules to alter the availability and catabolism of other substrates. Circulating FFA concentrations are substantially depressed upon ingestion of a ketone monoester, both at rest [65, 92] and during exercise [71, 121, 123]. Despite a reduced supply of FFA to skeletal muscle, fat oxidation is augmented during an exogenously-induced ketosis [71].

Whilst the mechanisms have not been elucidated, it is well observed that blood glucose concentrations are substantially lower following ketone supplementation, both at rest [65, 92] and during exercise [71, 112, 121, 123]. Importantly, this effect is also observed regardless of the individual's metabolic state (postprandial versus postabsorptive). In the postabsorptive state and during exercise - both conditions where endogenous glucose production is a significant contributor to maintaining blood glucose homeostasis [89, 124] - low circulating concentrations may result from a reduction in the supply of gluconeogenic amino acids to the liver [85, 86] (see Figure 1.4). However, this mechanism does not explain why blood glucose levels fall following ketone monoester ingestion in the postprandial period [91], when ele-

vated insulin concentrations mean that endogenous glucose production contributes minimally towards maintaining euglycaemia [89]. Whilst there is some evidence to suggest this may be caused by increased insulin secretion [90, 107], the observation that blood glucose concentrations fall following βHB infusion in type 1 diabetics [86] indicates the effect is insulin-independent.

Skeletal muscle glycolysis is suppressed during an exogenously-induced ketosis. The exercise-induced rise in blood lactate concentrations is decreased following ketone monoester ingestion [71, 112, 121, 123], as are post-exercise intramuscular glycolytic intermediates [71]. A reciprocal increase in fatty acid oxidation may occur, demonstrated by increased post-exercise intramuscular acyl-carnitine species and greater intramuscular triglyceride catabolism (measured through histological analysis of muscle samples) [71]. However, despite small but significant differences in blood lactate, recent work failed to observe a glycogen-sparing effect in athletes supplemented with a ketone monoester [123].

1.3.3 Effects of an exogenously-induced ketosis on acute exercise performance

Whilst there is strong evidence to suggest exercise metabolism is altered during an exogenously-induced ketosis, the subsequent effects on exercise performance are equivocal [125]. Six studies have reported the effects of ketone monoester ingestion on acute exercise performance (summarised in Table 1.4) with only two observing an improvement [71, 126]. Interpreting the ergogenic potential of an exogenously-induced ketosis is problematic due to the low number of studies reporting performance outcomes and high heterogeneity in the study protocols employed [125], including differences in: subjects' metabolic state (postabsorptive versus postprandial); timing of ketone monoester ingestion; dosing of ketone monoester and accompanying blood βHB concentrations; whether the ketone monoester is combined with CHO or bicarbonate (HCO_3^-) (the latter to compensate for the mild metabolic acidaemia resulting from ketone monoester ingestion, which is addressed in Chapter

3); the forms of exercise undertaken (running versus cycling); and the duration and intensity of exercise undertaken.

Exercise intensity may be a particularly important modulator of performance during an exogenously-induced ketosis [121]. Sustained muscular contraction necessitates that ATP is resynthesised at a rate that matches ATP demand. ATP resynthesis is ≈2-fold greater when driven by the glycolytic system compared to mitochondrial CHO oxidation and ≈4.5-fold greater versus mitochondrial fat oxidation [6]. Given this, at very high exercise intensities (for example, race-winning efforts) where ATP turnover is substantial, the inhibition of glycolysis during an exogenously-induced ketosis [71] may impede performance [121]. In part, this may explain the indifferent performance in studies employing exercise protocols that include high intensity efforts [112, 121].

Gaining a greater understanding of the factors regulating βHB metabolism in exercising skeletal muscle may help in forming dosing and usage strategies for the ketone monoester, which are currently lacking. This would facilitate testing the ergogenic potential of ketone supplementation under optimal conditions.

1.3.4 Effects of an exogenously-induced ketosis on recovery from exercise

Whilst most research has focused on the acute effects of an exogenously-induced ketosis on exercise physiology, metabolism and capacity, there is growing evidence that supplementing "gold-standard" nutritional guidelines with ketones may further enhance recovery from exercise.

Enhanced intramuscular glycogen synthesis

Pre-exercise intramuscular glycogen content is a strong determinant of endurance exercise capacity [33]. As such, replenishing spent stores is often a metabolic priority post exercise. Current recommendations are to consume 1.0 - 1.2 g·kg $BW^{-1}.h^{-1}$ CHO for four to six hours post exercise [127]. The provision of exogenous βHB with

Table 1.4: **Summary of studies reporting the effects of ketone monoester ingestion on acute exercise metabolism and performance in athletes.** * = work presented in Chapter 3 of this Thesis. Abbreviations not previously defined: KME = ketone monoester; PLA = placebo; TT = time trial

Reference	Intervention	Exercise protocol	Metabolic effects of KME	Performance effect
Cox et al. (2016) [71]	Postabsorptive; CHO alone or an isocaloric KME drink (573 mg·kg BW^{-1} + CHO)	1 h cycling ergometer exercise at 75% W_{Max} followed by a 30 min TT	βHB = ≈2 - 2.5 mM; ↓ blood lactate, glucose and FFA; ↓ intramuscular glycolytic intermediates, & ↑ acyl-carnitines, no change in TCA cycle intermediates; ↓ glycogen & ↑ triglyceride catabolism	Athletes cycled a significant (mean ± SEM) 411 ± 162 m further in the KME+CHO vs. CHO condition
Evans and Egan (2018) [112]	Postabsorptive; 1.2 g·min^{-1} of CHO with either 750 mg·kg BW^{-1} KME or PLA	Loughborough intermittent shuttle run test	βHB = ≈1 - 2.5 mM; ↓ blood lactate, glucose	Run time to exhaustion was similar between KME (mean [CI]; 229 s [178 - 280 s]) and PLA (268 s [199 - 336 s])
Dearlove et al. (2019) [121]*	Postabsorptive; 330 mg·kg BW^{-1} or a calorie free PLA	Cycle ergometer incremental test to volitional fatigue (3 min steps, 25 W ramp)	βHB = ≈2.5 - 3.5 mM; ↓ blood lactate, glucose and FFA; ↓ pH and HCO_3^-; ↓ workload at ventilatory threshold (T_{Vent}) and ↑ in workload at the fixed blood lactate accumulation (FBLA) of 4 mM	The W_{Max} achieved was similar between KME (mean ± SEM; 393 W ± 22 W) and control (389 W ± 20 W) conditions

Table 1.4 continued.

Reference	Intervention	Exercise protocol	Metabolic effects of KME	Performance effect
Evans et al. (2019) [122]	Postprandial; ≈1.0 g·min^{-1} of CHO with either 573 mg·kg BW^{-1} KME or PLA	1 h treadmill running at 65% $VO_{2\ Max}$ followed by a 10 km TT	βHB = ≈1 - 1.5 mM; no change in blood lactate, glucose, RER or running economy	Similar 10 km time in KME (mean [CI]; 2402 s [2204 - 2600 s]) and control (2422 s [2217 - 2628 s]) conditions
Poffé et al. (2020) [123]	Postprandial; CHO at 60 g·h^{-1} + KME (306 kilo calories (kcal) total) or a CHO control (29 kcal total)	3 h sub maximal intermittent cycling followed by a 15-min TT and "sprint"	βHB = ≈1 - 3 mM; ↓ blood glucose, lactate and FFA; ↓ blood pH, HCO_3^-; no difference in net muscle triglyceride and glycogen catabolism	Similar mean power during the 15 min TT (mean ± SD; KME: 273 ± 38; CHO: 272 ± 37 W) and "sprint" (KME: 59 ± 16; CHO: 58 ± 17 s)
Poffé et al. (2020) [126]	Postprandial; CHO at 60 g·h^{-1} with either: (i) KME (65 g total) + NaHCO3; (ii) KME (65 g total) + NaCl; (iii) PLA + NaHCO3; (iv) PLA + NaCl	3 h sub maximal intermittent cycling followed by a 15-min TT and "sprint"	βHB = ≈2 - 3 mM; KME = ↓ pH and HCO_3^-; KME + NaHCO3 = ↑ pH and HCO_3^-; ↓ blood glucose in ketosis conditions	Mean power output during the 15 min TT was ↑ ≈10 W in the KME + NaHCO3 vs. other conditions (p = 0.02 vs. KME)

gold-standard CHO feeding may further augment glycogen synthesis [107]. In highly trained athletes, ingesting ketone monoester before a 10 mM glucose clamp resulted in a 32% increase in infused glucose (an additional 61 g of glucose delivered over two hours) versus a CHO control, leading to an ≈2-fold increase in insulin secretion and a 50% increase in glycogen synthesis. This finding supports previous work in animal models that demonstrated greater intramuscular glycogen synthesis following the infusion of ketones [77, 128]. These data indicate that in the postabsorptive state: (i) reductions in blood glucose result from an increased ketone rate of disappearance (R_d), with skeletal muscle being a significant contributor to this effect; and (ii) ketone R_d from the blood is mediated by increased β cell insulin secretion. However, as discussed, elevated blood insulin concentrations are not consistently observed following either infusion or ingestion of ketones and are not necessary to lower blood glucose concentrations [86, 91, 121, 129–132]. Furthermore, van Dorne and colleagues [132] found that athletes fed CHO with a ketone monoester during post-exercise recovery had lower blood glucose but no change in intramuscular glycogen. Therefore, it appears that lower blood glucose concentrations during an exogenously-induced ketosis may be dissociated from skeletal muscle glucose uptake. In sum, it remains to be determined whether supplementing athletes with a ketone monoester post exercise is a useful nutritional strategy to stimulate glycogen supercompensation.

Increased muscle protein balance

The balance between muscle protein breakdown and synthesis determines net muscle protein balance (that is, muscle mass) [133]. A positive net muscle protein balance may be important for skeletal muscle repair and remodeling following exercise [134]. A pivotal metabolic adaptation to starvation is a progressive decrease in protein catabolism [47]. Evidence that amino acids, particularly alanine and leucine, are reduced during an exogenously-induced ketosis [83, 85–87] suggests that the reduction in muscle protein breakdown during starvation is, in part, mediated directly

by ketones. Muscle protein synthesis is mainly controlled by mammalian target of rapamycin complex 1 (mTORC1) signalling [135]. mTORC1 activity is increased in athletes supplemented with a ketone monoester post exercise, with subsequent *in vitro* studies attributing this effect to βHB, rather than other potentially confounding metabolic conditions such as hyperinsulinemia for altered blood FFA concentrations [132]. Taken together, these results indicate that ketone supplementation could favourably effect net muscle protein breakdown in the post-exercise period and thus potentially aid recovery from, and adaptations made to exercise.

Prevention of sympathetic over training

Supplementing athletes with βHB following exhaustive exercise appears to diminish the sympathetic nervous response to training overload, as observed by a relative reduction in nocturnal catecholamine excretion [136]. The mechanism of this effect has not been established but is plausibly mediated through βHB antagonism of FFAR3 [96] (see paragraph 1.2.4). This suggests that ingesting βHB during periods of high-intensity exercise training could be useful for the prevention of sympathetic overtraining syndrome [137]. This effect was credited for improvements (albeit, limited) in exercise performance in athletes consuming the ketone monoester versus a lipid containing control drink [136].

Altered gene expression?

Physiological concentrations of βHB inhibits histone deacetylase (HDAC)1, HDAC3, and HDAC4 (classes I and IIa) [49, 138], proteins that regulate gene expression by deacetylating histone lysine residues. Histone hyperacetylation is typically associated with conformational changes associated with gene expression [139]. It follows, therefore, that βHB-mediated suppression of HDACs would be expected to increase transcriptional activity. In the context of adaptations to exercise training, of particular interest is that class IIa HDACs repress the transcriptional activity of myocyte enhancer factor 2 (MEF2) [140], a transcription factor that regulates the

expression of slow myosin heavy chain and mitochondrial proteins in cardiac and skeletal muscle [141]. Furthermore, class IIa HDAC phosphorylation (resulting in nuclear exclusion) has been identified as a key regulator of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) expression [142], a transcription factor that modulates mitochondrial biogenesis and fat metabolism [143]. βHB also directly post-translationally modifies histones at lysine residues (lysine β-hydroxybutyrylation) in fasted or diabetic mouse liver, which is associated with activating fasting-related gene networks [144]. The collective actions of βHB on gene expression may, therefore, help facilitate the transition from the fed to fasted state by increasing peripheral tissues' capacity for the mitochondrial oxidation of fat. Notably, these signalling pathways overlap with those activated through endurance exercise training [36]. However, further work is required to elucidate the effects of βHB signalling in human skeletal muscle, and whether ketone supplementation may augment favourable adaptive responses to exercise.

1.3.5 Summary

Ingesting ketones creates a unique metabolic state, distinctly different to the metabolic conditions associated with the endogenous synthesis of ketones following starvation or ketogenic diet. Supplementing athletes with a ketone monoester appears to mimic potentially advantageous aspects of starvation metabolism: increasing fat oxidation, "sparing" (or impairing) glycogen catabolism, whilst simultaneously providing a novel substrate for working skeletal muscle. However, whether this translates to increased exercise performance remains to be determined. Finally, ketone monoester supplementation post-exercise may augment recovery by enhancing anabolic processes, including both glycogen synthesis and muscle protein synthesis.

1.4 Therapeutic application of exogenous ketones

Dysfunctional metabolism has a central role in many of the non-communicable diseases affecting modern Western society [145]. Given the discussed effects of ketones on human metabolism, there is substantial interest in the therapeutic utility of ketone supplementation [17, 109, 146]. For example, an exogenously-induced ketosis could provide a useful adjunctive therapy to treat the hyperglycemia and hyperlipidemia associated with insulin resistant states. Furthermore, the combination of protein sparing and reduced sympathetic tone during ketosis may mean that ketone supplementation is a useful metabolic intervention for catabolic disorders, such as sepsis-related muscle atrophy. It is beyond the scope of this Introduction to detail the full therapeutic potential of an exogenously-induced ketosis (see [17, 109, 146] for comprehensive reviews). However, it is important to state that studies of exercise physiology have provided invaluable insights into the mechanisms underlying human health and disease [147] and as such, work in this Thesis may facilitate the targeted therapeutic application of exogenous ketosis.

1.5 This Thesis

Work presented in this Thesis sought to deliver novel insights into the effects of an exogenously-induced ketosis on human physiology and metabolism, and thus provide new understandings regarding its potential to enhance human physical performance. In particular, the questions addressed within this work were:

Chapter 3

- What are the effects of an exogenously-induced ketosis (and the associated ketoacidaemia) on the physiological response to incremental-intensity exercise and how does this affect athletes' perceptions of exertion?

Chapter 4

- To what extent does exogenous βHB contribute to energy expenditure at rest and during exercise?
- Are exogenous βHB oxidation rates affected by the intensity of exercise performed and the concentration of blood βHB?
- Does the acute supplementation of βHB enhance exercise efficiency?

Chapter 5

- Can chronically elevating blood βHB through ingestion of a ketone monoester drink modulate the physiological and metabolic adaptations made in response to exercise training?
- Does supplementing athletes with a ketone monoester drink enhance exercise training capacity?

Chapter 2

General methods

2.1 Ethical considerations and principles

All studies were reviewed and given favourable ethical opinions by the regional Oxford Research Ethics Review Committee (OXREC) as part of the following applications:

- 15/SC/0542 (applicable to work undertaken in Chapters 3 and 4);
- 17/SC/0297 (applicable to work undertaken in Chapter 5).

All work conformed to the standards detailed in the Declaration of Helsinki. Participants provided written, informed consent before undertaking study procedures.

2.2 Subjects and recruitment

Participants were recruited through OXREC-approved advertisements or direct invitation to participate. Subjects were required to meet all of the following inclusion criteria:

- aged 18 - 45 years;
- healthy;
- currently undertaking a minimum of 6 hours of exercise training per week;
- have been training continuously for a minimum of 3 months prior to enrolment;

- and be free of injury or illness.

Potential participants were not considered for enrolment if they met any of the following criteria:

- history of cardiovascular, neuromuscular, endocrine or neurological illness, or any other medical condition adjudged to compromise the subjects' safety or research goals;
- currently taking medications that may interfere with lipid or glucose metabolism;
- current smoking;
- females not taking the oral contraceptive pill (due to the variability in hormonal patterns and substrate levels at different stages of the menstrual cycle [148]);
- pregnancy or current breastfeeding;
- inability to provide informed consent.

2.3 Ketone drinks

For all studies, nutritional ketones were delivered in the form of a ketone monoester (D-β-hydroxybutyrate-R 1,3-butandiol monoester; TΔS, UK) diluted in water. The ketone monoester delivers 4.8 kcal·g^{-1}. The ketone monoester has been designated as generally regarded as safe by the Food and Drug Administration (FDA) and has been approved for use by the World Anti-Doping Authority (WADA). The safety and toxicology [114, 149], and the pharmacokinetics of the ketone monoester have been published [116]. Specific study drink formulas are provided within the Methods sections for each Chapter.

2.4 Testing procedures

2.4.1 Baseline exercise test protocol

For work presented in Chapters 4 and 5, baseline exercise tests were undertaken to determine athletes' W_{Max} and power at $VO_{2\ peak}$. In Chapter 3, the protocol described here was used as the exercise intervention. All exercise was performed on an electronically braked cycling ergometer (Ergoselect 100, Ergoline, Germany). Before commencing exercise, participants adjusted the saddle height and handlebar position according to personal preference. Bike geometry was recorded and kept constant for all subsequent study visits. Participants wore a comfortable fitting face mask attached to an indirect calorimeter for the duration of tests (see paragraph 2.4.2, below). Participants could monitor their cadence on a digital display, which they were instructed to maintain between 80 - 100 revolutions per min (RPM). The ergometer was set to the RPM-independent mode, meaning the workload was held constant during each 3-min interval regardless of the cadence held. Tests began at 100 W (low intensity) and increased 25 W every 3 min until volitional fatigue. Furthermore, one of the following criteria must also have been met to satisfy that exercise was undertaken to exhaustion:

- an inability to maintain a cadence of $\geq$60 RPM;
- the maximum HR attained was within 10 beats per min (BPM) of an age predicted maximum (220 - age);
- the maximum RER attained was $\geq$1.0.

Respiratory gases were measured continuously through indirect calorimetry. $VO_{2\ peak}$ was defined as the highest volume of oxygen consumed (VO_2) during the test. W_{Max} was calculated according to the formula [150]:

$$W_{Max} = W_{Step\ N\text{-}1} + (time\ elapsed/180\ sec \cdot 25\ W) \quad (2.1)$$

2.4.2 Cardiorespiratory measurements

Indirect calorimetry

Indirect calorimetry was used for the determination of volume of carbon dioxide expelled (VCO_2), VO_2 (allowing the subsequent calculation of RER) and ventilatory exchange (V_E). Participants wore a snug, comfortable fitting face mask (Hans Rudolph, USA) attached to an indirect calorimeter (Metalyzer 3BR2, Cortex Biophysik, Germany) for the continuous sampling of respiratory gases, displayed in real time using Metasoft® (v7.9.1, Cortex Biophysik, Germany).

Following the completion of measurements, cardiorespiratory data was time averaged (5 sec) in MetaSoft® and exported to Excel® (Microsoft Corporation, USA) for further analysis. Clearly aberrant values were omitted, and a minimum 30 sec average was taken for each measurement point.

A three-step calibration of the indirect calorimeter was performed prior to all tests. First, atmospheric pressure was measured and entered into MetaSoft®. A two-point gas calibration was then performed (ambient air versus a calibration gas: 15% O_2, 5% CO_2; Cranlea Human Performance, UK). Finally, a 3 L volume syringe (Hans Rudolph, USA) was used to calibrate gas flow through the pneumotachograph turbine.

Based on the known stoichiometry of substrate oxidation, the contribution of fat and CHO to overall energy expenditure can be predicted by VO_2 and VCO_2 measurements [20]. The inclusion of the a third, potentially major substrate (ketones) confounds these equations (see Table 2.1 for a list of RER values for various substrates). Adjustments have been proposed to account for the effects of ketogenesis and ketolysis during an endogenous ketosis [20]; however, these have not been validated for the unique physiological state of an exogenously-induced ketosis. Thus, whilst RER values are presented within this Thesis, caution must be exerted when interpreting them.

Table 2.1: **RER values for major energy substrates**

	RER
Palmitate	1.0
Glucose	0.7
βHB	0.89
AcAc	1.0

Heart rate

Subjects wore a HR monitor chest strap (T31, Polar Electro, Finland) that communicated directly to the Metalyzer unit via a receiver cable attached to the bicycle ergometer.

Respiratory gas sampling

Respired gases were collected for the measurement of substrate oxidation using stable isotope methodologies for work undertaken in Chapters 4 and 5. Participants breathed into a mouthpiece containing a one-way valve, connected to a 50 L Douglas bag. Duplicate samples of respired gases were then collected in 12 mL Exetainer™ vials (Labco, UK) and stored at room temperature until further analysis (see Methods sections in Chapters 4 and 5 for details of the analysis procedures).

2.4.3 Blood sampling

Arterialised venous blood sampling

Arterialised venous blood samples were taken for work in Chapters 3 and 4. A 22-gauge catheter (BD Venflon™ Pro Safety, BD, USA) was inserted retrogradely into a dorsal vein of the hand. A 3-way tap (BD Connecta™ Plus Stopcock, BD, USA) was attached, allowing for repeated blood sampling. To achieve arterialised venous values, participants hands were heated for 2 min before blood was drawn [151]. The catheter was flushed with ≈1 mL sodium chloride (Injection BP 0.9% w/v, Kent Pharmaceuticals, UK) after each sample was taken.

Venous blood sampling

Venous blood samples were taken for work presented in Chapter 5. A 22-gauge catheter (BD Venflon™ Pro Safety, BD, USA) was inserted into the median cubital or cephalic vein of the antecubital fosa. A three-way tap (BD Connecta™ Plus Stopcock, BD, USA) was attached, allowing for repeated blood sampling. The catheter was flushed with ≈1 mL sodium chloride (Injection BP 0.9% w/v, Kent Pharmaceuticals, UK) after each sample was taken.

2.4.4 Percutaneous tissue sampling

Skeletal muscle and subcutaneous adipose tissue biopsies were performed on participants for work in Chapters 4 and 5.

Skeletal muscle biopsy

A site in the lower third of the vastus lateralis muscle was cleaned with 0.5% chlorhexidine spray (Hydrex®, Ecolab, UK) and prepared. Vastus lateralis was chosen due to its mixed fibre composition [152] and its heavy recruitment during cycling exercise [153]. Between 1 - 2 mL of local anaesthetic (1% Lidocaine hydrochloride without adrenaline, Hameln Pharmaceuticals, UK) was injected, ensuring good dermal infiltration. A small incision was made through the dermis and deep fascia with a scalpel blade (No. 11, Swann Morton®, UK). A 14-gauge spring-loaded, automatic biopsy device (Achieve™ biopsy needle 14 G x 6 cm, UK Medical, UK) was inserted through the incision and into the muscle (Figure 2.1). Four passes were made, each yielding ≈20 - 30 mg muscle. The direction of the biopsy needle was changed for each pass so that samples were taken ≈4 cm apart. This method has been shown to prevent the resampling of previously biopsied (damaged) fibres [154]. Samples were immediately transferred from the biopsy needle to an Eppendorf tube and frozen in liquid nitrogen (within ≈30 sec of sampling). Samples were stored at -80 °C until further processing.

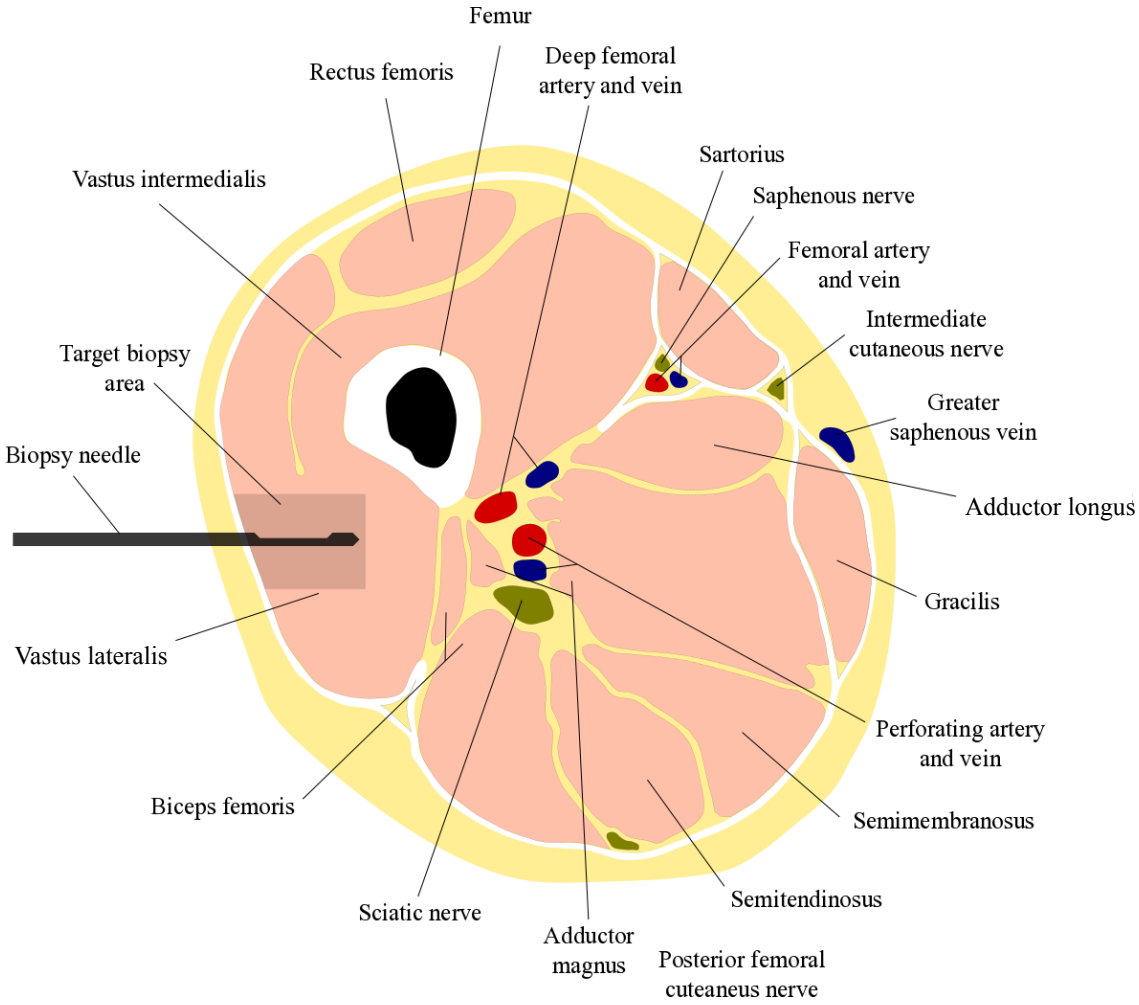


Figure 2.1: **Incision of biopsy needle into the lateral thigh.**

Subcutaneous adipose tissue biopsy

A site lateral and inferior to the umbilicus was cleaned with 0.5 % chlorhexidine spray (Hydrex®, Ecolab, UK) and prepared. Approximately 1 mL of local anaesthetic (1% Lidocaine hydrochloride without adrenaline, Hameln Pharmaceuticals, UK) was injected, ensuring good dermal infiltration. A small incision was made through the dermis using a scalpel blade (No. 11, Swann Morton®, UK). A 14-gauge needle (14 G x 8 cm, B. Braun Medical, UK) attached to a 10 mL syringe (BD Plastipak 3-Part Luer Slip Syringe, BD, USA) containing 3 mL of saline (Injection BP 0.9% w/v, Kent Pharmaceuticals, UK) was inserted through the incision. The syringe plunger was withdrawn to create suction, and a "sawing" motion applied to disrupt and collect adipocytes held within the fascia. Once ≈100 mg of adipose tissue was attained,

each sample was washed with saline (Injection BP 0.9% w/v, Kent Pharmaceuticals, UK), transferred to an Eppendorf tube and frozen in liquid nitrogen (within ≈2 min of sampling). Samples were stored at -80 °C until further processing.

2.5 Analytical procedures

2.5.1 Muscle fibre phenotyping

In Chapter 4, the percentage of slow-oxidative fibres in skeletal muscle biopsy samples was determined using methods adapted from Koopman and colleagues [155]. Frozen vastus lateralis samples were embedded in optimal cutting temperature (OCT) compound (Tissue-Tek® OCT™ Compound, Labtech, UK), sectioned (4 μm) using a Cryostat Microtome (OTF5000, Bright Instruments, UK) and mounted onto slides (SuperFrost® Plus, VWR International, UK). Samples were fixed for 1 hour using a 4% formaldehyde solution. Triton X-100 (0.5%) was used to make samples permeable, before being blocked for 1 hour at room temperature (1% bovine serum albumin, 0.1% Triton X-100). Samples were incubated overnight with a primary antibody (product code: ab11083, anti-slow skeletal myosin heavy chain, Abcam, UK) diluted in the block solution (1:50). The primary antibody was internally validated on dissected rat soleus (high percentage of slow-oxidative muscle fibres [156]) and anterior tibialis (high percentage of fast-glycolytic muscle fibres [156]) muscle (see Appendix A) and has previously been validated in human skeletal muscle [157]. The secondary antibody (product code: F0257, anti-Mouse igG (whole molecule)-fluorescein isothiocyanate (FITC) produced in goat, Merck and Co., USA) diluted in the block solution (1:200) was applied for 1 hour at room temperature. Finally, 4',6-diamidino-2-phnylindole dihydrochloride (DAPI) (1:1000 dilution in double distilled water) was added for 10 min to detect nuclei, before a coverslip was applied.

Slides were imaged using a DeltaVision widefield fluorescence imaging system (GE Healthcare, USA) consisting of a 10x/0.4 NA objective lens (Olympus Life

Science, UK) fitted to an Olympus IX71 inverted microscope stand. A Lumencor SpectraX LED light engine (Lumencor, USA) with excitation lines at 395 nm and 470 nm and appropriate emission filters were used to sequentially image DAPI and FITC fluorochromes on a CoolSNAP HQ2 CCD camera (Photometrics, USA). The instrument was driven using the softWoRx software package (GE Healthcare, USA) and subsequent image analysis performed in ImageJ (NIH, USA). Quantification of slow-oxidative fibres was performed through manual counting (minimum of 50 fibres) and expressed as a percentage of total muscle fibres.

2.5.2 Metabolomics

Metabolite profiles were determined in pre- and post-exercise muscle samples in Chapter 5, as a method to determine experimental intervention effects on exercise substrate metabolism.

Metabolite extractions

Protocol optimisation experiments were performed in rat skeletal muscle tissue, as described in Appendix B. Two muscle biopsy samples were used per measurement point, yielding an average tissue mass of 38.0 ± 5.4 mg. An 80% methanol buffer (250 μL) was added to samples, which were then homogenised for ≈30 sec (Ultra-Turrax® T25, IKA-Werke, Germany). The homogenate was filtered through centrifugation (1400 RPM, 4 °C for 14 min). Nucleic acid concentration (double strand deoxyribonucleic acid (DNA)) was quantified in the filtrate using a Nanodrop ultraviolet visible spectrophotometer (Thermo Fisher Scientific, USA). Final solutions were prepared by diluting samples in the 80% methanol buffer, such that all contained 20 ng·μL DNA^{-1} (the lowest recorded nucleic acid concentration). A 25 μL sample was taken from each of the final solutions and combined to create a quality control sample.

Sample analysis and data processing

Samples were analysed at the Department of Chemistry, The University of Oxford, using four separate methods [158, 159] and two different liquid chromatography systems (Dionex^{TM} ICS-5000+ Capillary HPIC^{TM} System and a Ultimate^{TM} 3000 UHPLC system (both, Thermo Fisher Scientific, USA). These methods were:

- Ion exchange chromatography;
- C18 reversed phase analysis of underivatised samples;
- C18 reversed phase analysis of derivatised samples;
- Hydrophobic-interaction liquid chromatography-mass spectrometry.

Each system was coupled directly to a Q-Exactive^{TM} UHMR mass spectrometer with a heated electrospray ionization probe (Thermo Fisher Scientific, USA). Raw data files were processed using Progenesis® QI (Waters, UK). This process included alignment of retention times, peak picking by identification of the presence of natural abundance isotope peaks, characterising multiple adduct forms and identification of metabolites using an in-house database. Retention times, accurate mass values, relative isotope abundances and fragmentation patterns were compared between authentic standards and the samples measured. Identifications were accepted only when the following criteria were met:

- $\leq$5 ppm differences between measured and theoretical mass (based on chemical formula);
- $\leq$30 sec differences between authentic standard and analyte retention times;
- isotope peak abundance measurements for analytes were $\geq$90% matched to the theoretical value generated from the chemical formula.

Where measured, fragmentation patterns were matched to the base peak and two additional peak matches in the mass spectrometry $(MS)^2$ spectrum to within 12

ppm. In instances where metabolites were reported in duplicate (due to the multiple MS methods employed) the identification with the greatest measured intensity was retained. Identified ions with a measured intensity of zero, or that had a CV>30%, were also deleted. Ions with measured intensity <500 counts were assessed by referencing of retention time error, isotope similarity and mass error. Normalised glycolytic, TCA cycle and carnitine compound values were subsequently analysed in Prism v8.3 (GraphPad Software, USA).

2.5.3 Proteomics

Untargeted (label-free) proteomics was performed on skeletal muscle biopsy samples for work in Chapter 5.

Sample digestion and purification

Frozen skeletal muscle samples (mean ± SD mass, 19.5 ± 0.5 mg) were homogenised in lysis buffer (75 mM tris(hydroxymethyl)aminomethane (tris)-hydrochloric acid (HCl) pH 6.8, 3.8% sodium dodecyl sulfate (SDS), 4 M urea, 20% glycerol) using a Precellys 24 tissue homogeniser (Bertin Instruments, France). The homogenate was heated for 5 min at 90 °C, centrifuged (13,000 g for 5 min at 4 °C) and the supernatant removed. Protein concentrations were quantified in the supernatant (mean ± SD concentration, 13.2 ± 0.3 μg/μL) using a bicinchoninic acid assay (product number: 23227; Thermo Fisher Scientific, USA). Finally, 5% β-mercaptoethanol was added to samples, which were then placed on a heating block for 5 min at 90 °C. Samples were subsequently stored at -80 °C.

Samples were thawed on ice. A volume containing 100 μg protein was diluted in the lysis buffer, minus the SDS, for each sample (final sample volume of 50 μL). A 1,4-Dithiothreitol (DTT) reducing agent was added to samples (final concentration 5 mM; 200 mM DTT in 0.1 M tris buffer, pH 7.8), which were then incubated at room temperature for 60 min. An iodoacetamide alkylating reagent was then added (final concentration 20 mM; 200 mM iodoacetamide in 0.1 M tris buffer, pH

7.8) followed by another 60 min incubation at room temperature. The total sample volume was increased to 200 μL with double distilled water. Methanol (600 μL) and chloroform (150 μL) were added to samples before vortexing. Double distilled water (450 μL) was added, and samples were again vortexed. Samples were then centrifuged (12000 g at room temperature for 1 min) and the upper aqueous phase was removed and discarded. Sample precipitation steps were repeated. Methanol (450 μL) was then added to the remaining organic phase, which was subsequently vortexed and centrifuged for 2 min (12000 g at room temperature). The supernatant was removed, and the remaining protein pellets were resuspended in 50 μL urea buffer (6 M urea in 0.1 M tris buffer, pH 7.8) by vortexing and sonification. Double distiled water (250 μL) was added to reduce the urea concentration to <1 M. Trypsin was added in a 1:50 ratio in relation to the total protein content of samples. Samples were gently mixed and placed on a heat block set at 37 °C for overnight digestion.

The following day, Sep-Pak C18 purification was performed. Sep-Pak columns were attached to a vacuum manifold. Columns were flushed with 500 μL of "solution B" (65% CH_3CN, 35% MiliQ-H_2O, 0.1% trifluoroacetic acid). "Solution A" (1 mL; 98% MiliQ-H_2O, 2% CH_3CN, 0.1% trifluoroacetic acid) and the peptide digest samples were added to columns, which were then slowly flushed. Columns were washed with solution A. Finally, samples were eluted with 600 μL of solution B. The collected eluate was then dried down in a speed vac and resuspended in 20 μL of solution A.

Sample analysis and data processing

Samples were analysed at the Target Discovery Institute, The University of Oxford, using liquid chromatography-coupled mass spectrometry. Peptides were separated on an EASY-Spray column ES803 and analysed on a Dionex UltiMate^{TM} 3000 UHPLC system and an Orbitrap Fusion^{TM} Lumos^{TM} platform (both Thermo Fisher Scientific, USA) [160]. Raw data were imported into Progenesis® QI (Waters, UK) using default parameters. Tandem MS (MS^2) data were searched using Mascot

(v.2.5, Matrix Science, UK) against the Universal Periodic Review human database. Mass tolerances were set to 10 ppm for precursor and 0.5 Da for fragment masses. Peptide-level false discovery rate (FDR) was adjusted to 1%. Peptides with a score of <20 were discarded. Data were centered and normalised in Progenesis® QI (Waters, UK), before being extracted for further data processing in Perseus (Max-Plank Institute of Biochemistry).

Proteomics data analysis

Differences in protein abundance (delta protein abundance; abundance in post-intervention muscle samples - abundance in baseline muscle samples) were analysed using Perseus, a platform developed for the interpretation of proteomics data [161]. The workflow employed followed best-practice principles for the analysis of proteomics data via Perseus [162]. All identified proteins with peptide counts or unique peptides $\leq$2 were removed. Keratins (contaminants) were excluded and data were filtered so that a minimum of 70% of values were present in all groups. Histograms were created to check data distribution before statistical analyses were applied (described within Chapter 5).

2.5.4 Determination of intramuscular triglyceride content

The methods used followed the protocol described by Mehlem and colleagues [163]. Briefly, frozen vastus lateralis samples were embedded in OCT compound (Tissue-Tek® OCT™ Compound, Labtech, UK), sectioned (12 μm) using a Cryostat Microtome (OTF5000, Bright Instruments, UK) and mounted onto slides (SuperFrost® Plus, VWR International, UK). Slides were subsequently stored at -80 °C. Staining was performed in batches, ensuring paired samples were within the same batch.

Samples were thawed at room temperature for 10 min. An area was traced around the sections using a liquid blocking pen. A working solution of oil red o (ORO) was applied for 10 min ($\approx$250 μM), ensuring the sections were fully immersed. Samples were then rinsed under running tap water for 30 min and air dried for $\approx$15

min. Slides were mounted using a water-soluble mounting medium (ProLong™ Gold Antifade Mountant, Thermo Fisher Scientific, USA) and coverslips were applied. Finally, the mounting medium was left to dry for ≈10 min and the coverslips edges were sealed with nail polish.

Slides were imaged on the day of staining with an Olympus Fluoview® FV3000 Confocal Laser Scanning Microscope (Olympus, Japan) using a TexasRed excitation filter (540 - 580 nm). Images were subsequently analysed using ImageJ (NIH, USA). First, images were converted to grayscale and the region of interest tool was used to draw around whole muscle fibres/collections of whole muscle fibres (damaged fibres were not included within regions of interest). A minimum of 50 muscle fibres were analysed. Pixel intensity thresholds were then applied (lower threshold = 55, upper threshold = 255; see example grayscale image of post-threshold sample in Figure 2.2). Finally, the mean pixel intensity for regions of interest on each sample was determined, which was used to quantify intramuscular triglyceride content.

2.5.5 Gene expression

Gene expression analyses were performed on subcutaneous adipose tissue biopsy samples for work in Chapter 5. Frozen subcutaneous adipose tissue samples were homogenised in Tri-reagent (product code: AM9738, Ambion, USA) and ribonucleic acid (RNA) was extracted using a standard Tri-reagent protocol. Complementary DNA (cDNA) was synthesised from 500 μg of total RNA using the High Capacity cDNA Reverse Transcription Kit (product code: 4366596, Thermo Fisher Scientific, USA). Quantitative polymerase chain reaction (qPCR) was performed in triplicate using a 1/40 cDNA dilution with Taqman™ Assays (Thermo Fisher Scientific, USA) and Kapa Probe Fast Mastermix (product code: KK4706, Merck and Co., USA). Reactions were run in 384-well plates (product code: PCR0430, Scientific Lab Supplies, UK) on an ABI Prism 7900HT (Thermo Fisher Scientific, USA). The relative expression of target transcripts was calculated using the delta C_t method [164] (2.2,

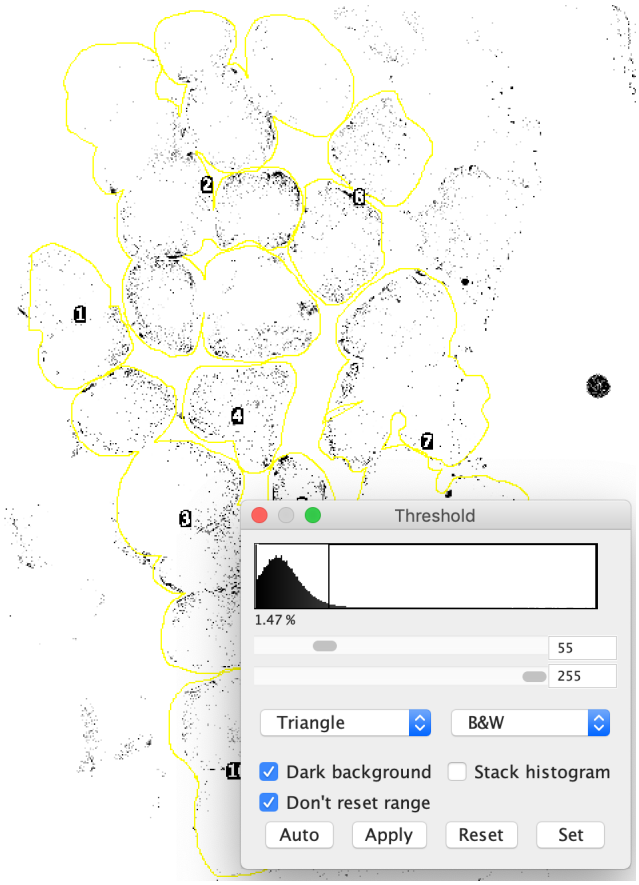


Figure 2.2: **Grayscale post-threshold image of pixel intensity used to determine intramuscular triacylglycerol content.** Yellow outlines represent the measured regions of interest.

below).

$$\Delta C_t = Efficiency^{\ Calibrator\ Ct\ -\ sample\ Ct} \tag{2.2}$$

Delta C_t values for target transcripts were normalised to the delta C_t (geometric mean) of the selected reference transcripts [165]: Importin 8 (Hs00914057 m1), peptidylprolyl isomerase A (PPIA) (Hs99999904 m1) (performed twice) and TATA box (Hs00427620 m1) (see Figure 2.3).

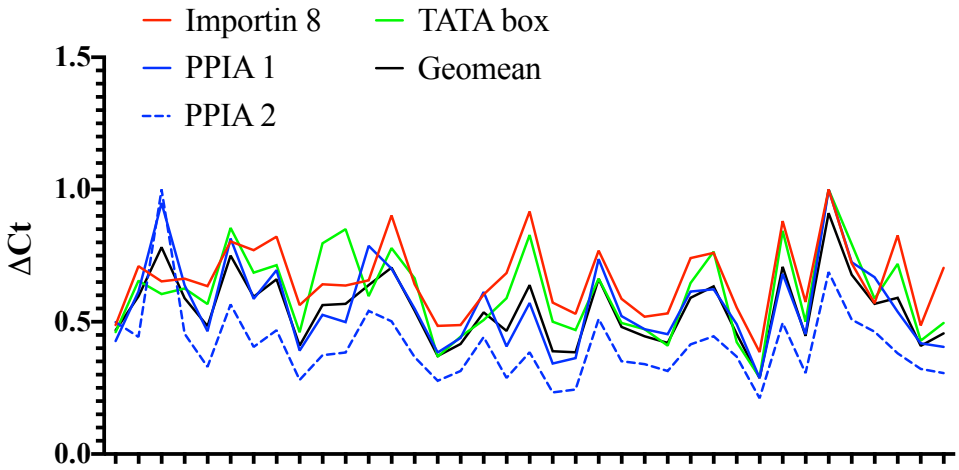


Figure 2.3: **qPCR housekeeping transcript delta C_t values.**

2.6 Statistics

Throughout the Thesis, significance is determined as $p<0.05$ and data is presented as mean ± SD. The statistical methods employed are described in detail within Chapter Methods/Results sections. Assumptions for parametric testing, including normality of distribution and homogeneity of variance, were tested before undertaking analyses. Where data did not meet these assumptions, log10 transformations were applied (all analyses were performed on the transformed data, but non-transformed data are presented in Figures and Results) or appropriate non-parametric tests were employed.

Chapter 3

The effects of an exogenously-induced ketosis on acid-base physiology and perceptions of exercise exertion during incremental intensity exercise

3.1 Introduction

The physiological response to incremental intensity exercise under "normal" metabolic conditions is well described [166–168]. As exercise intensity increases, a greater contribution from glycolysis to overall energy expenditure leads to increased intra- and extra-cellular H^+ concentrations [12]. Typically, this coincides with a non-steady state rise in blood lactate levels [169]. In response, a coordinated chemical and respiratory compensation occurs. Within blood, the accumulation of H^+ drives the carbonic acid equation (3.1) towards the production of CO_2 + H_2O.

$$CO_2 + H_2O \Longleftrightarrow H_2CO_3 \Longleftrightarrow H^+ + HCO_3^- \tag{3.1}$$

H^+ simultaneously excites peripheral chemoreceptors, which stimulates exercise hyperventilation [170]. This is observed as a non-linear inflection in the relationship

between V_E and VO_2, termed the T_{Vent} [168]. As a consequence, blood CO_2 content falls, resulting in a partial correction of blood pH [168].

Supplementing athletes with ketones may alter this well observed physiological response to incremental-intensity exercise. Glycolysis is impaired during an exogenously-induced ketosis [48, 71], thus reducing metabolically derived H^+ [12] and blood lactate concentration. Additionally, consumption of the ketone monoester causes a fall in blood pH and HCO_3^- [92, 123], replicating the mild ketoacidosis observed with starvation [171] or a ketogenic diet [172]. Importantly, the effects on acid-base balance are mild compared to those observed during the pathological ketoacidosis of poorly controlled type 1 diabetes, where blood ketone concentrations may exceed 25 mM and pH may fall below 6.9 [173–175]. Nonetheless, inducing even small decreases in pre-exercise blood pH may subsequently alter ventilatory dynamics during exercise [176, 177]. However, the effects of an exogenously-induced ketosis on the physiological response to incremental intensity exercise have not been determined.

Blood H^+ concentration is an important humoral signal that shapes sensations of muscular fatigue [178–181] and ventilatory exertion [182] during exercise. It is possible, therefore, that perceived exertion may be heightened by a mild ketoacidosis. Previous work found that supplementing athletes with βHB had no effect on ratings of perceived exertion (RPE) during both steady state [112, 123] and intermittent intensity exercise [122]. However, to investigate subtle but potentially important differences in perceived exertion, it may be useful to measure components of RPE [183]. This may include location of exertion - at a basic level, legs versus lungs [183, 184] - and the emotional response to these [183, 185–187].

Therefore, work in this Chapter sought to: (i) describe the human physiological response to incremental intensity exercise during an exogenously-induced ketosis; and (ii) determine whether supplementing athletes with βHB altered components of perceived exertion and emotional responses to these.

3.2 Methods

3.2.1 Subjects and recruitment

Twelve healthy, athletically trained subjects participated in this single-blind, cross-over design (two conditions), random-order-controlled study. Subjects were recruited from advertisements at local sports clubs or via direct invitation to participate. Participant characteristics are presented in Table 3.1. Subjects were actively participating in either cycling, running, triathlon or swimming. Inclusion and exclusion criteria matched those presented in paragraph 2.2.

Table 3.1: **Participant characteristics.** $VO_{2\ peak}$ and W_{Max} values presented here represent scores achieved at the control study visit.

	Mean (SD)
Gender (m/f)	9/3
Age (yr)	28.0 (5.7)
Height (m)	1.85 (0.12)
Weight (kg)	77.8 (12.0)
BMI ($kg \cdot m^{2\text{-}1}$)	22.6 (1.1)
Training $h \cdot wk^{-1}$	13.6 (6.0)
VO_2 peak ($L \cdot min^{-1}$)	4.4 (0.7)
Weight adjusted VO_2 peak ($mL \cdot kg^{-1}$)	56.5 (3.9)
W_{Max} (W)	382.3 (69.8)

3.2.2 Study visits

Participants were asked to refrain from vigorous exercise and alcohol consumption for 48 hours prior to study visits. Participants attended the Department of Physiology, Anatomy and Genetics (DPAG) at ≈0800 in the overnight fasted state (Figure 3.1). Two baseline (t = -60 min; "pre-drink") arterialised venous blood samples were drawn (see paragraph 2.4.3 for a description of the sampling technique): (i) for the immediate analysis of blood gases (≈1 mL); and (ii) for the analysis of serum substrate and insulin concentrations (≈3 mL). The second sample was stored in an

ice-water bath for the duration of the visit. Cardiorespiratory measures were made via indirect calorimetry (see paragraph 2.4.2) and participants scored their resting exertion (scales described below) and gastrointestinal discomfort (scales described in [119]). Finally, subjects consumed either a βHB containing drink (330 mg·kg BW^{-1} ketone monoester diluted in 400 mL of water, target blood βHB concentration of ≈3.5 mM; mean ± SD calories = 123.3 ± 19.0) or a control drink (bitter flavoured water) before resting for 60 min. Based on a mean participant weight of 77.8 ± 12.0 kg, the mean amount of βHB delivered was 25.7 ± 3.96 g (123.4 ± 19.0 kcal).

Baseline measurements (described above) were repeated before participants commenced exercise (t = 0 min). Exercise commenced at 100 W and increased 25 W every 3 min until participants reached fatigue (see paragraph 2.4.1 for the criteria used to determine fatigue). This is a validated protocol for determining changes in respiratory gas parameters during exercise in response to experimental interventions [188]. Blood samples were taken, and any gastrointestinal distress was measured, in the last minute of the 100 W, 200 W and 300 W intervals, and immediately upon exhaustion (W_{Max}). Cardiorespiratory measures were taken in the last minute of the 100 W, 150 W, 200 W, 250 W and 300 W intervals, and at W_{Max}. Perceived exertion was measured in the final 30 sec of all intervals and at W_{Max}. Room temperature and airflow were consistent across study visits. Participants consumed water ad libitum. In males, the presence of a female observer may cause a significant decrease in reported subjective perceptions of exertion [189]. As such, we ensured that there was at least one male and one female study investigator present for all visits. Participants were given verbal encouragement by study investigators.

3.2.3 Blood gas analysis

Whole blood samples (≈1 mL) were immediately analysed using a benchtop blood gas analyser (ABL800, Radiometer UK, UK). Attained pH and HCO_3^- values were subsequently corrected using a custom MATLAB script (v2017a, MathWorks, USA), which accounted for the increased haemoglobin (Hb) saturation in arterialised blood

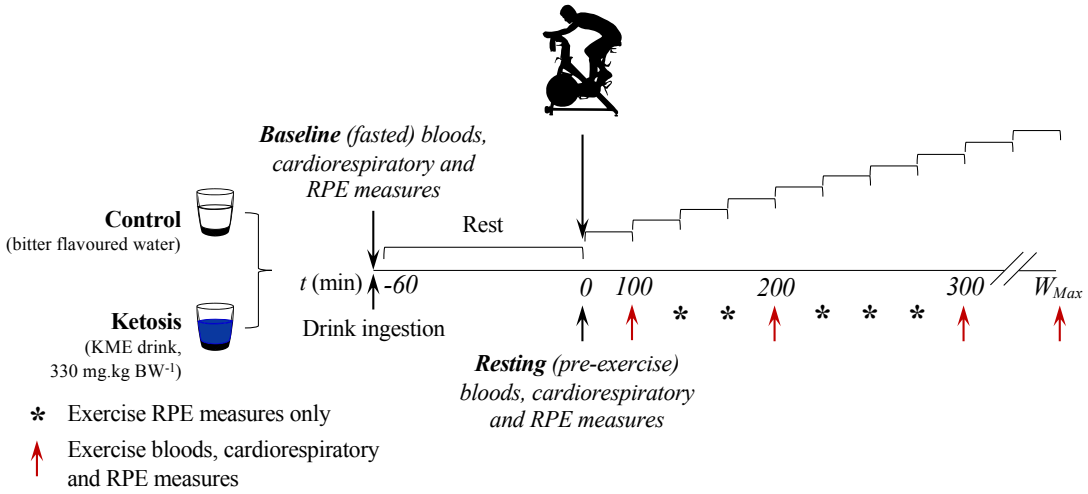


Figure 3.1: **Study visit protocol.** Highly-trained athletes (n=12) attended the laboratory on two occasions to determine the effects of an exogenously-induced ketosis on the physiological response to incremental intensity exercise and perceptions of exercise exertion.

(see Appendix C for a description of the calculations used). Anion gaps were calculated as previously described [190].

3.2.4 Blood substrate and insulin analyses

βHB was analysed immediately from whole blood samples using a portable analyser (Freestyle Optium Neo, Abbott Laboratories, USA). Following completion of the visit, samples kept on ice were centrifuged for 10 min at 3600 RPM. The plasma component was aliquoted into Eppendorf tubes and these were stored at -80 °C until later analysis. Frozen samples were thawed at room temperature for ≈30 min before biochemical analyses were undertaken. Serum glucose, non-esterified fatty acids (NEFA) and lactate were determined using a calibrated, semi-automated benchtop analyser (Pentra C400, Horiba Medical, France). Serum insulin was assayed using an enzyme-linked immunosorbent assay (ELISA) kit (product code: 10-1113-01, Mercodia, Sweden). All analyses were performed in duplicate, with the average value taken.

3.2.5 Determinations of lactate accumulation, ventilatory threshold and exercise capacity

The FBLA of 4 mM was used as a measure of lactate accumulation in response to incremental-intensity exercise. This was assessed with a freely available software add-in for Excel (V16.32, Microsoft®, USA) [191]. The T_{Vent} was calculated using the V-slope method [168]. A second, blinded investigator verified all T_{Vent} estimates. In the event of significant discordance (defined as a $\geq$5% difference in evaluations), a third, blinded investigator acted as arbiter. Exercise capacity was determined as W_{Max} (calculated as described in paragraph 2.4.1).

3.2.6 Determination of perceived exertion

The present work sought to determine the effect of an exogenously-induced ketosis on compartmentalised perceptions of RPE (leg discomfort and breathlessness discomfort) and the concurrent emotional responses to these perceptions (anxiety related to leg discomfort and anxiety related to breathlessness). As such, subjective scores of RPE ("What is your perceived exertion?"), breathlessness ("How breathless are you?"), leg discomfort ("What is your leg discomfort?"), anxiety of breathing ("How anxious are you about your breathing?") and anxiety of leg discomfort ("How anxious are you about your leg discomfort?") were recorded in a random order on a linear scale of 0 – 10 (0 = none to 10 = maximum).

3.3 Statistics

All data are reported as mean ± SD and statistical significance was taken as $p<0.05$. Blood, cardiorespiratory and perceived exertion data were analysed using 2-way repeated measures (RM) analysis of variance (ANOVA) (factors: condition (two levels) and exercise intensity (6 - 12 levels, depending on the outcome measure)) with appropriate post-hoc corrections applied (Prism v8.3, GraphPad Software, USA).

Simple linear regressions were used to test within condition associations between workloads at the FBLA of 4 mM and the T_{Vent}. Between condition comparisons of W_{Max} and workloads corresponding to the FBLA and the T_{Vent} were assessed using Student's paired t-tests.

Physiological factors contributing to observed differences in ventilation and perceived exertion were investigated using mixed effect modelling. A general linear mixed effect regression (GLMER) model was fitted to V_E and each exertion measure identified as being altered during ketosis (R v3.4.1, The R Foundation for Statistical Computing, Austria) [192, 193]. Models contained a subject-specific random effect, thus allowing a different model intercept to be fitted for each subject. Two participants were excluded from the modelling analyses due to incomplete data sets (missing HR values). All variables were centered and scaled prior to inclusion.

Global models were fitted containing hypothesised contributing variables. Parsimonious models were then created through a process of backward elimination of non-significant terms. In order to preserve a hierarchical, well-formulated model, non-significant variables were included if their interaction with another variable was significant [194]. To determine whether the reduced set of variables impaired the model fit, reduced models were then tested against the initial global models using the ANOVA function in R and type II (no interaction terms) or III (interaction present) Wald chi-square tests. Full model R^2 and fixed effect R^2 values for both global and reduced models were calculated using the r2beta function within the r2glmm package, employing a standardised variance approach [195]. Finally, the predicted data was plotted against the measured data for visualisation of model accuracy.

3.4 Results

3.4.1 Blood βHB concentration

Blood βHB rose to 3.7 ± 0.3 mM following ingestion of the ketone monoester drink ($p<0.001$) and remained significantly elevated versus the control condition through-

out exercise (all $p<0.001$, Figure 3.2). Notably, blood βHB levels fell from rest (post-drink) to 100 W ($p=0.018$) and 100 W to 200 W ($p<0.001$) in the ketosis condition, but thereafter plateaued at higher exercise intensities. Ingestion of the ketone monoester was not associated with increased gastrointestinal distress (data not shown).

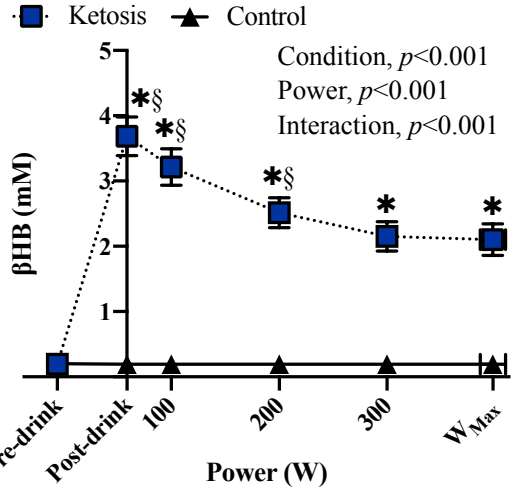


Figure 3.2: **Blood βHB concentration.** * = significant difference between control and ketosis conditions. § = significant difference versus the previous measurement in the ketosis condition.

3.4.2 Blood gases, electrolytes and the anion gap

Blood pH was similar at baseline in ketosis (7.40 ± 0.18) and control (7.41 ± 0.01) conditions (Figure 3.3 A). Following ingestion of study drinks, blood pH fell significantly versus control to 7.37 ± 0.01 ($p=0.003$) and remained significantly lower during exercise at 100 W ($p=0.007$) and 200 W ($p<0.001$). Consequently, blood HCO_3^- was significantly lower versus the control condition at rest (post-drink; $p=0.012$) and during exercise at 100 W, 200 W (both $p<0.001$) and 300 W ($p=0.006$; Figure 3.3 B). No differences were observed in blood potassium, sodium, calcium and chloride (data not shown). The anion gap was lower in the ketosis versus control condition at rest (post-drink), 100 W (both, $p<0.001$), 200 W ($p=0.011$) and 300 W ($p=0.008$; Figure 3.3 C). Condition differences in the anion gap disappeared when βHB anions were accounted for.

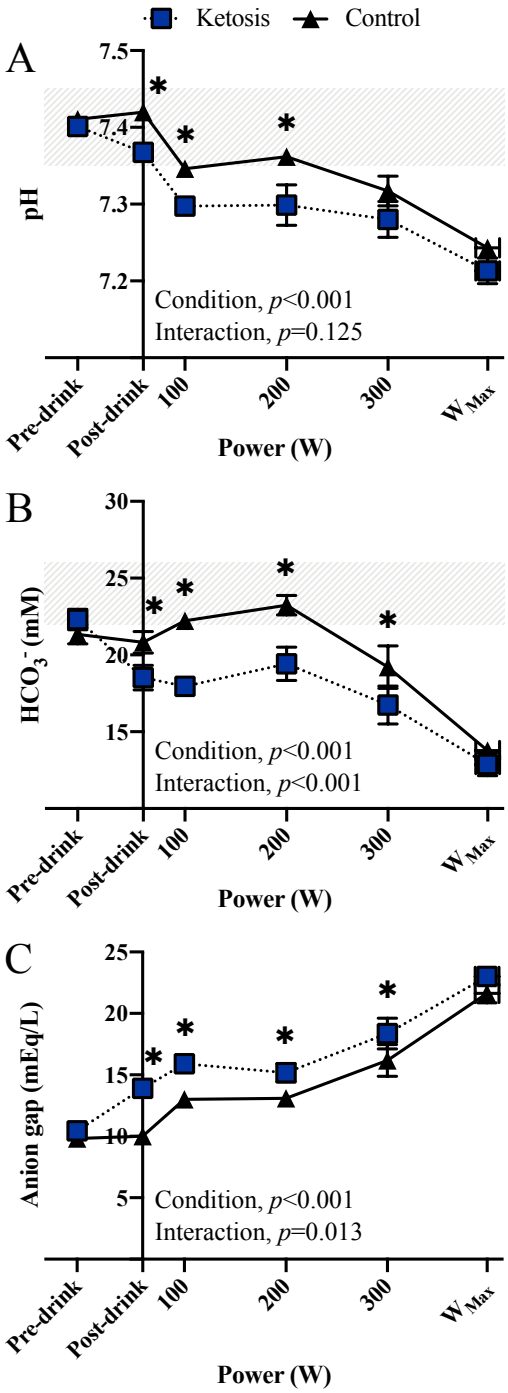


Figure 3.3: **Blood gases and the anion gap.** Blood **(A)** pH and **(B)** HCO_3^- concentration at rest and during exercise in the ketosis and control conditions. **(C)** Anion gaps at rest and during exercise in the ketosis and control conditions. * = significant difference between control and ketosis conditions. Shaded areas in (A) and (B) represent normal resting ranges.

3.4.3 Cardiorespiratory measures

HR was similar in ketosis and control conditions (Figure 3.4 A). There was a main effect of condition for V_E, which was significantly greater during ketosis (p=0.034;

Figure 3.4 B). Partial pressure of end tidal carbon dioxide ($PETCO_2$) was significantly lower at 100 W (p<0.001), 150 W (p=0.009) and 200 W (p=0.045; Figure 3.4 C) during ketosis. VO_2 tended to be lower during ketosis (ANOVA condition effect, p=0.091) with no between-condition differences in VCO_2 (Figures 3.4 D and E). Consequently, there was a main effect of condition for RER (p=0.036), which was higher during ketosis (Figure 3.4 F). The factors driving changes in ventilation were then investigated through a GLMER model (Figure 3.5). Parsimonious model data are presented below (see Appendix D for results of the full models).

Significant, positive effects of power (p<0.01, R^2=0.60), HCO_3^- (p<0.01, R^2=0.26), potassium (K^+) (p<0.01, R^2=0.12), HR (p<0.01, R^2=0.11) and sex (male) (p<0.01, R^2=0.11) were found to drive ventilation. A significant negative effect was observed for H^+ (p<0.01, R^2=0.34) and the interaction effect between power and HR (p<0.01, R^2=0.37). The reduced model explained 98% of the variability in ventilation.

3.4.4 Blood substrates and insulin

Blood lactate concentration was significantly lower in the ketosis versus control condition at 300 W (5.5 ± 2.1 mM versus 7.0 ± 3.2 mM, respectively; p<0.001) and W_{Max} (10.8 ± 1.2 mM versus 13.3 ± 0.8 mM, respectively; p<0.001) (Figure 3.6 A). Blood glucose and NEFA were significantly lower at rest (post-drink) and throughout exercise in the ketosis versus control condition (Figures 3.6 B and C). There were no differences in blood insulin concentrations between conditions (Figure 3.6 D).

3.4.5 Lactate accumulation, ventilatory threshold and exercise capacity

Power output at the FBLA of 4 mM was higher in the ketosis (323 ± 65 W) versus control (298 ± 75 W) condition (p=0.032) (Figure 3.7 B). An approximately equal-in-magnitude and opposite-in-direction effect was observed for power output at the T_{Vent}, which was significantly lower during ketosis (238 ± 13 W) versus control (260

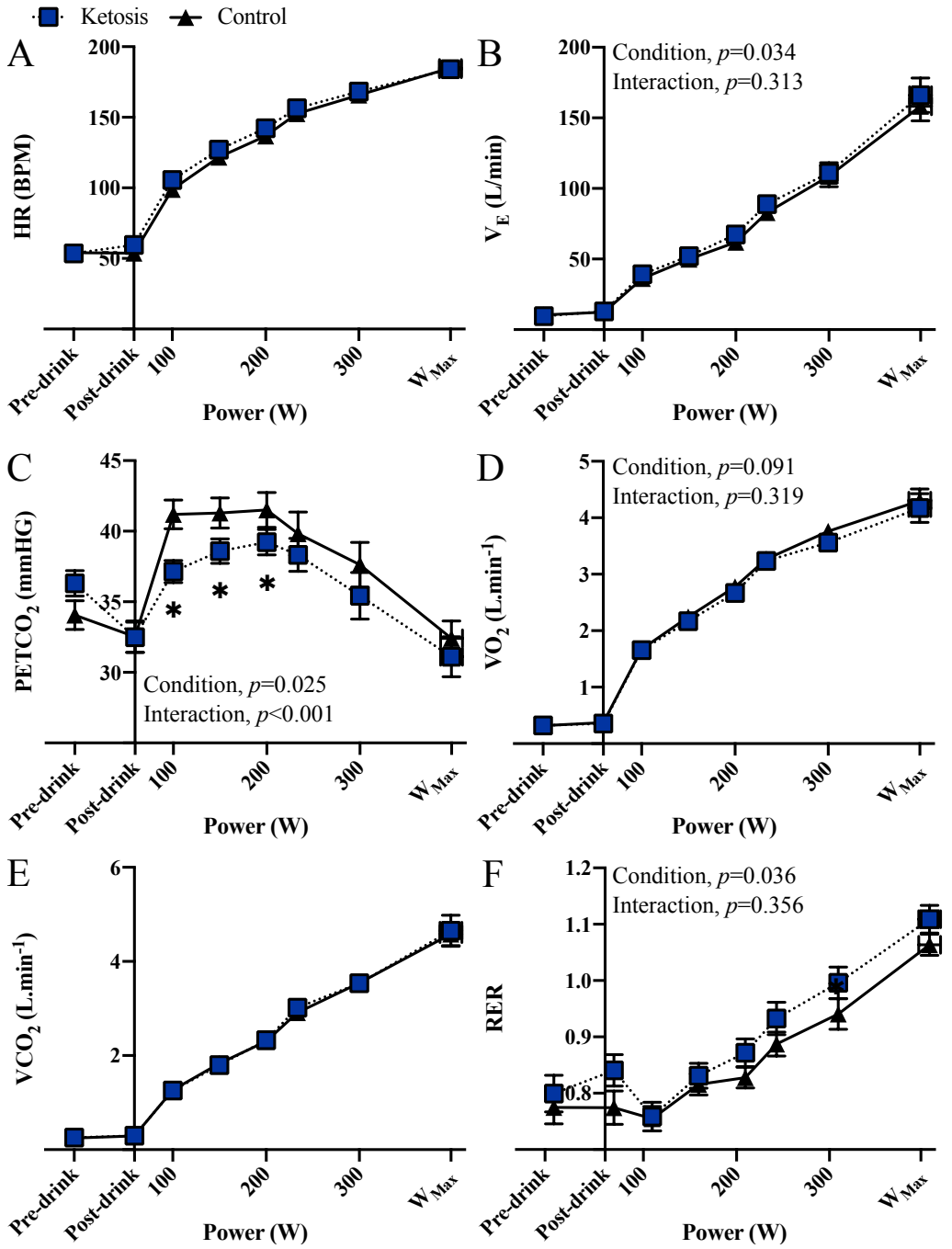


Figure 3.4: **Cardiorespiratory measures.** **(A)** HR; **(B)** V_E; **(C)** $PETCO_2$; **(D)** VO_2; **(E)** VCO_2; and **(F)** RER at rest and during exercise in the control and ketosis conditions. * = significant difference between control and ketosis conditions.

± 15 W) (p=0.012) (Figure 3.7 C). Power outputs at the FBLA of 4 mM and the T_{Vent} were significantly positively correlated in the control (R^2=0.680, p=0.003) but not ketosis (R^2=0.364, p=0.065) conditions (Figures 3.7 D and E). Exercise capacity (W_{Max}) was similar in ketosis (393 ± 22 W) and control (389 ± 20 W) conditions (Figure 3.7 A).

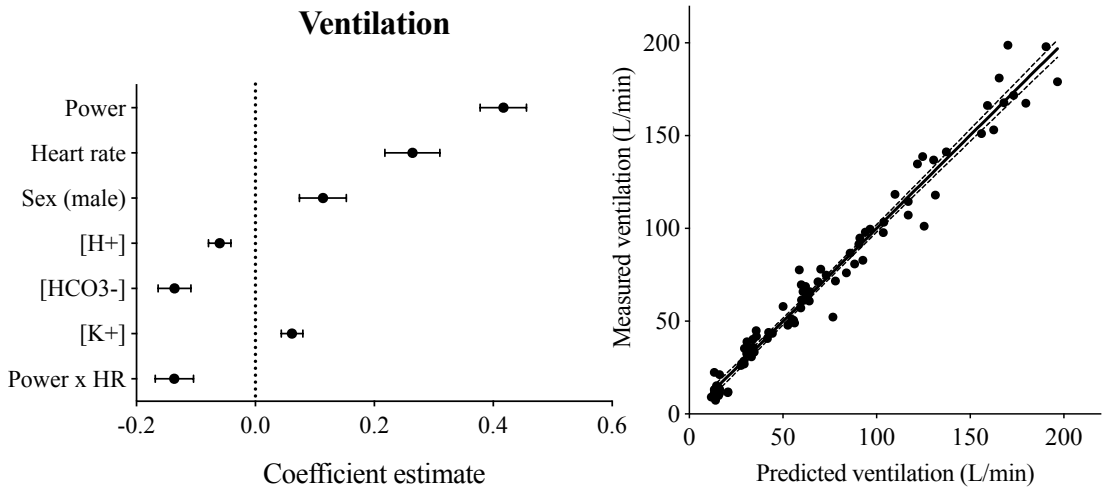


Figure 3.5: **Factors contributing to ventilation.** Summary of model coefficients (left) and predicted versus measured outcomes for the GLMER models (right).

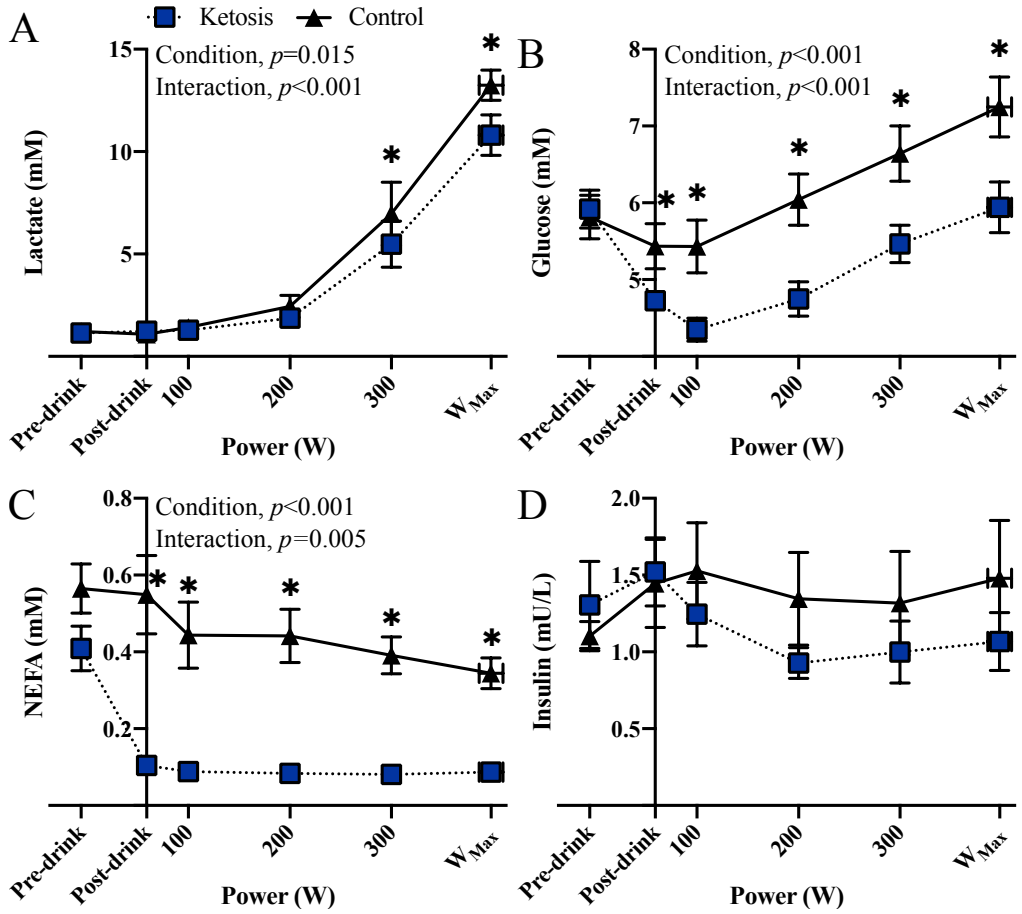


Figure 3.6: **Blood substrates and insulin.** Blood **(A)** lactate; **(B)** glucose; **(C)** NEFA; and **(D)** insulin concentrations at rest and during exercise in the ketosis and control conditions. * = significant difference between control and ketosis conditions.

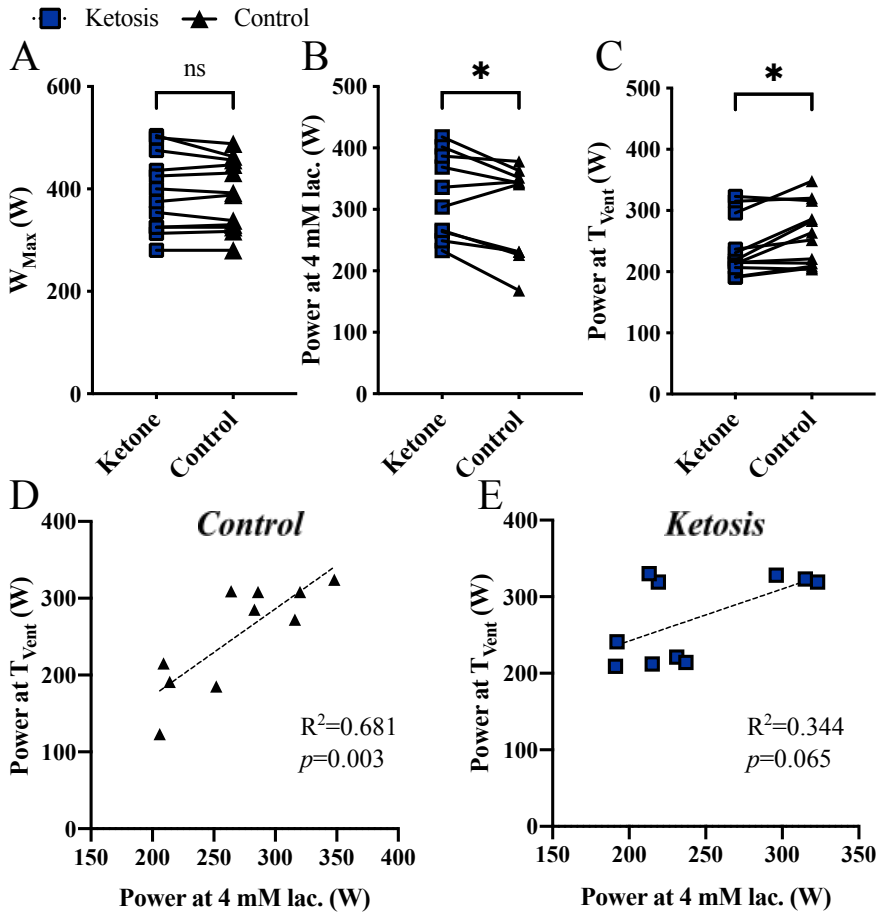


Figure 3.7: **Physiological markers and exercise capacity.** **(A)** Exercise capacity (W_{Max}) in the control and ketosis conditions. Power output at **(B)** the FBLA of 4 mM and **(C)** the T_{Vent} in the control and ketosis conditions. Associations between power at the FBLA of 4 mM and the T_{Vent} in the **(D)** control and **(E)** ketosis conditions. * = significant difference between control and ketosis conditions.

3.4.6 Perceived exercise exertion

The effect of ketosis on RPE, compartmentalised perceptions of RPE and emotional responses to these perceptions were initially analysed by 2-way RM ANOVA. RPE was similar in ketosis and control conditions (Figure 3.8). There was a main effect of condition for athletes' perception of leg discomfort, which was greater during ketosis (p<0.001; Figure 3.9 A). A condition-power interaction effect was also present (p=0.024), with leg discomfort being significantly greater during ketosis at 200 W. There was a corresponding main effect of condition for athletes' anxiety related to leg discomfort, which was increased during ketosis (p=0.005; Figure 3.9 B). The perception of breathlessness was similar in control and ketosis conditions (Figure

3.9 C); however, there was a main effect of condition for breathing anxiety, which was significantly increased in the ketosis condition (p=0.031; Figure 3.9 D).

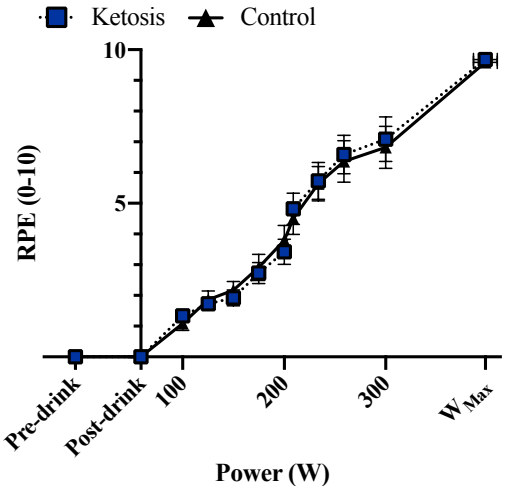


Figure 3.8: **Ratings of perceived exertion.** Measured at rest and during exercise in the control and ketosis conditions.

The factors contributing to altered perceptions of leg discomfort and emotional responses to leg discomfort and breathing were then investigated through a series of GLMER models (Figure 3.10). Parsimonious model data are presented below (see Appendix E for results of the full models).

Leg discomfort

Significant, positive effects of power (p<0.01, R^2=0.20), HR (p=0.01, R^2=0.11) and H^+ (p<0.01, R^2=0.18) were found to contribute to leg discomfort scores. Additionally, there was a significant negative effect for the interaction between power and H^+ (p<0.01, R^2=0.22) (Figure 3.10 A). The reduced model explained 90% of the variability in leg discomfort.

Anxiety of leg discomfort

Significant, positive effects of HR (p<0.01, R^2=0.08), H^+ (p<0.01, R^2=0.06), lactate (p<0.01, R^2=0.01) and the interaction between power and sex (male) (p=0.01, R^2=0.02) were found to contribute to anxiety of leg discomfort (Figure 3.10 B).

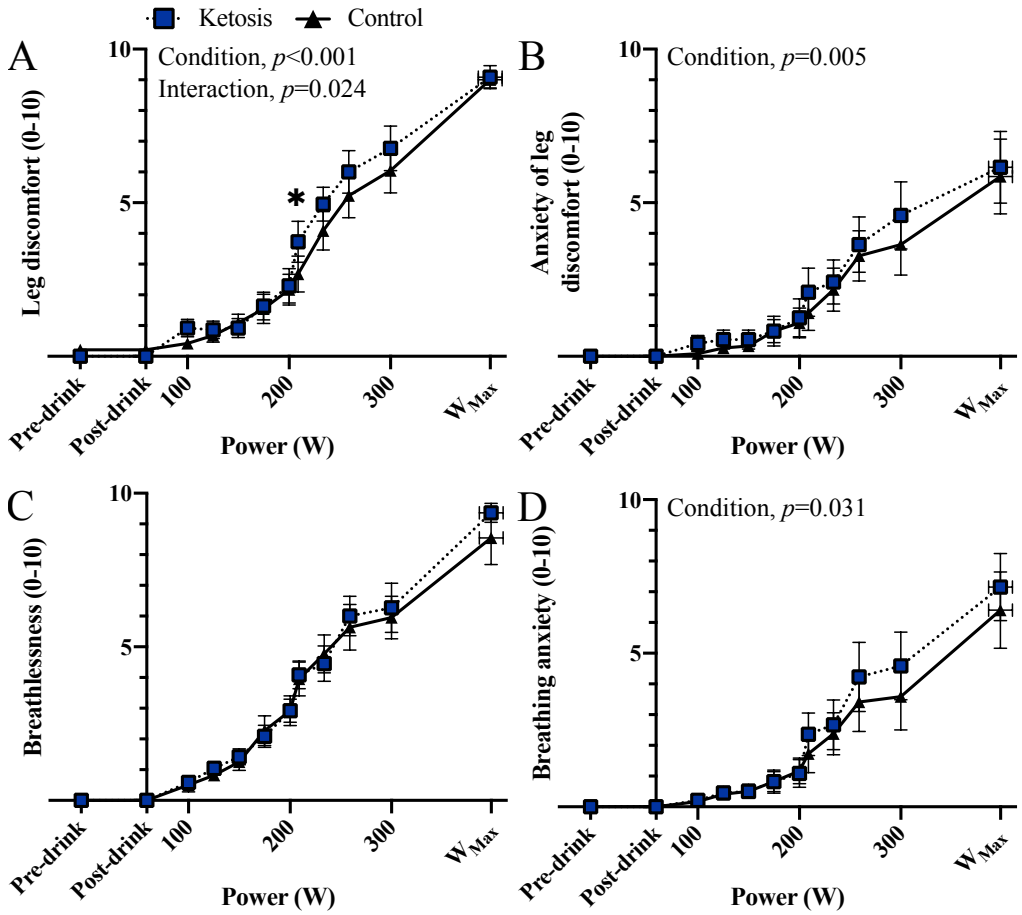


Figure 3.9: **Compartmentalised perceptions of exertion and emotional responses to these perceptions.** **(A)** Leg discomfort; **(B)** anxiety related to leg discomfort; **(C)** breathlessness; and **(D)** anxiety related to breathing at rest and during exercise in the control and ketosis conditions. * = significant difference between control and ketosis conditions.

βHB (p=0.05, R^2=0.01) and glucose (p=0.04, R^2=<0.01) and the interaction between HR and sex (male) (p=0.01, R^2=0.06) and power and H^+ (p<0.01, R^2=0.14) had significant negative effects. The reduced model explained 81% of the variability in anxiety of leg discomfort.

Anxiety related to breathing

There were significant positive effects of power (p<0.01, R^2=0.53) and H^+ (p<0.01, R^2=0.16), and a significant negative effect for the interactions between power and H^+ (p<0.01, R^2=0.19) towards breathing-related anxiety (Figure 3.10 C). The reduced model explained 74% of the variability in anxiety of breathing.

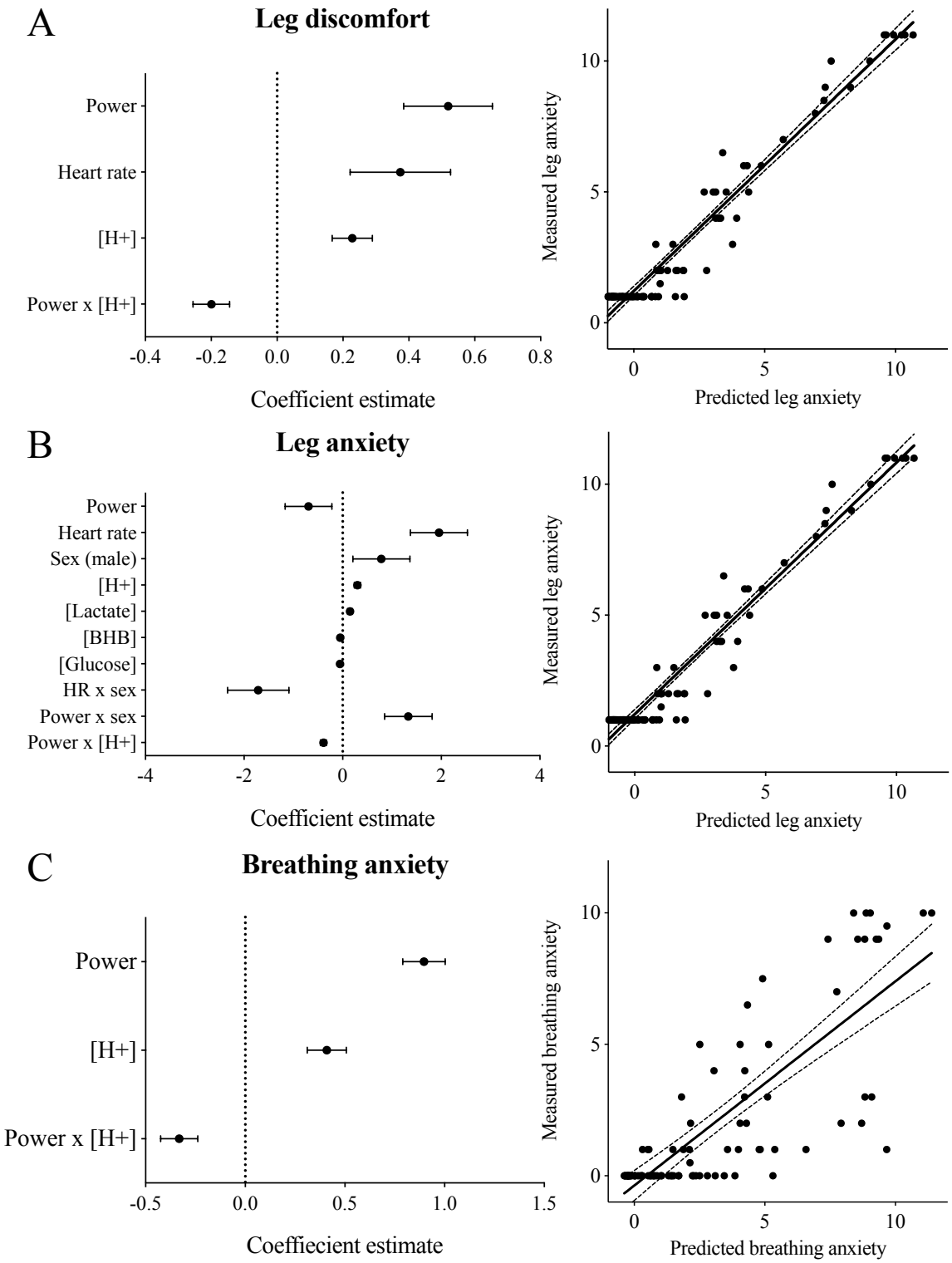


Figure 3.10: **Factors contributing to perceptions of leg discomfort, leg anxiety and breathing anxiety.** **(A)** Leg discomfort; **(B)** anxiety related to leg discomfort; and **(C)** anxiety related to breathing at rest and during exercise in the control and ketosis conditions. Summary of model coefficients (left) and predicted versus measured outcomes for the GLMER models (right).

3.5 Discussion

This study sought to describe the human physiological response to incremental intensity exercise during an exogenously-induced ketosis, and to determine whether the resulting mild ketoacidosis would alter perceptions of exercise exertion. The main findings were: (i) ingestion of a ketone monoester caused a small but significant fall in blood pH; (ii) athletes compensated for a mild ketoacidaemia through increased ventilation and earlier exercise-induced hyperventilation; (iii) there was a rightward shift in the blood lactate curve during ketosis, meaning lactate accumulation was dissociated from the T_{Vent}; and (iv) primarily due to the increased acid load, compartmentalised perceptions of RPE and emotional responses to these perceptions were heightened during ketosis.

3.5.1 Metabolic and respiratory compensations for a mild ketoacidaemia

Consumption of the ketone monoester drink caused blood pH and HCO_3^- to fall and increased the anion gap at rest and during submaximal exercise intensities. The anion gap was similar between conditions when βHB anions were accounted for, indicating that perturbations to acid-base balance were caused by ketoacidaemia. As a consequence, V_E was elevated ≈5% during ketosis. Additionally, exercise-induced hyperventilation, observed as an inflection in the relationship between V_E and VO_2 [168, 196], occurred at a 22 W lower power output. Increased ventilation was sufficient to reduce $PETCO_2$ (an acid producing blood gas), which was lower during ketosis at 100 W, 150 W and 200 W (low to moderate exercise intensities). Notably, blood $PETCO_2$ had returned to control levels by 300 W, simultaneous with the convergence of blood pH values.

Physiological measures predicted to contribute to changes in ventilation were entered into a combined generalised linear model. Within this model, we observed that exercise intensity, HR and sex (male) were strong positive determinants of

ventilation, and as such had significantly positive coefficients. Generalised linear models only assign the unique variance accounted for by each regressor within each coefficient. Blood H^+ has high collinearity with other measures included in the model, such as exercise intensity and HR. Thus, the coefficient allocated to H^+ was significantly negative. In other words, having accounted for the more linear effects induced by increasing exercise intensity on ventilation, the relationship between the remaining variance explained by H^+ with ventilation was negative. This negative relationship was likely caused by hyperventilation at higher exercise intensities and a subsequent partial correction in blood H^+ concentration (see equation 3.1) [166, 168]. Of the blood metabolites included in the model, HCO_3^- explained the greatest proportion of variance in ventilation (26%). Interestingly, K^+ had a significant, independent effect on V_E. This finding corroborates previous reports showing close associations between blood K^+ concentration and V_E [197, 198] during exercise and highlights its potential importance as a humoral ventilatory stimulator [199, 200].

Blood lactate accumulation was lower during ketosis, as demonstrated by 25 W increase in power output at the FBLA of 4 mM and a rightward shift in the blood lactate curve. These findings are consistent with previous observations of reduced glycolytic flux following βHB supplementation [71]. The βHB-mediated suppression of glycolysis, an acid producing pathway [12], may also have contributed to the convergence of blood pH values at high exercise intensities. The concept that increased glycolytic flux causes exercise hyperventilation is a central tenet of the anaerobic threshold model [168], commonly used to quantify exercise tolerance in athletes and for pre-surgery risk stratification in clinical cohorts [201]. The observation that lactate accumulation was dissociated from exercise-induced hyperventilation suggests the anaerobic threshold model is not applicable during an exogenously-induced ketosis.

3.5.2 Perceptions of exertion are altered during ketosis

Despite widespread changes in physiology, RPE was unaffected by ketosis. This finding is consistent with previous reports that have used a Borg scale [202] to measure exertion during an exogenously-induced ketosis [112, 122, 123] and provides further support for a dominant component of anticipatory feedforward (central) contributions to perceived exertion [203–205]. RPE represents the amalgamated score of numerous exercise-related symptoms [184]. Of these, "local" muscle pain (in other words, specific to the muscle(s) being recruited) and "central" breathlessness are the largest contributors [206]. Either one, or a combination of the two, may cause the cessation of exercise [207]. Furthermore, RPE may not encompass affective emotions such as anxiety, which also influence exercise capacity [183, 208, 209]. Thus, the present work sought to determine the effect of ketoacidosis on compartmentalised perceptions of exertion (legs versus lungs) and emotional responses to these perceptions. Anxiety of breathing, leg discomfort and anxiety of leg discomfort were all increased during ketosis. Blood H^+ concentration was a significant, independent contributor to all these measures, thus implicating ketoacidosis as the cause. Anxiety of leg discomfort was a particularly complex measure, including significant negative contributions of glucose and βHB. Both are potentially important substrates for working muscle [18, 57, 71] and the brain [55, 210, 211] during exercise. Thus, it is plausible that a compromised availability of these fuels may cause heightened anxiety of leg discomfort.

This finding may provide a clue regarding the evolutionary advantage of ketoacidosis. Starving humans are not idle; even when energy stores are challenged, the need to forage, hunt and avoid predation necessitates performing potentially vigorous physical activity [212]. In this setting, an increased awareness regarding the metabolic "cost" of physical activity may be advantageous, thus potentially avoiding futile and possibly life-threatening energy expenditure. That said, W_{Max} was almost identical when athletes were supplemented with a ketone monoester versus a calorie-free control drink. Thus, in the present work, the heightened perception

and anxiety of exercise exertion did not appear to impede athletes exercise capacity (that is, change their behaviour). However, recent work has demonstrated that supplementing athletes with the ketone monoester and sodium bicarbonate (NaHCO3) (thus increasing the blood buffering capacity and preventing ketoacidaemia) significantly increased exercise performance versus the ketone monoester alone [126], indicating that ketoacidaemia may modulate exercise capacity.

3.5.3 Limitations and future directions

Several methods have been proposed to measure blood lactate accumulation during incremental intensity exercise [213]. A major criticism of the FBLA approach (as used here) is that inter-individual comparisons can be misleading; blood lactate concentration at the lactate threshold (the inflection point in blood lactate accumulation [168]) are wide, ranging from 1.4 mM to 7.5 mM [214]. However, the present study performed within-subject comparisons (by nature of the cross-over design), thus negating the major criticism of the approach employed. Additionally, the FBLA method reduces the interpolation required to identify the lactate threshold and thus, may limit bias.

Whilst this study has yielded interesting preliminary modelling results on perceptions of exercise exertion, large scale data sets with a more equal gender balance and diverse study population would be required to support our results.

3.6 Conclusions

Athletes compensate for a mild ketoacidosis through greater ventilation and an earlier exercise-induced hyperventilation. Athletes supplemented with βHB had increased perceptions of leg discomfort, and increased anxiety related to leg discomfort and breathing. Changes in perceptions of exercise exertion were primarily driven by the elevated blood H^+ concentration during ketosis.

Chapter 4

The effect of blood βHB concentration and exercise intensity on exogenous βHB oxidation rates

4.1 Introduction

βHB oxidation in exercising skeletal muscle may have thermodynamic advantages versus other substrates, which could enhance exercise efficiency [17, 69, 70] (see paragraph 1.2.3). Per mole C_2 units, the complete oxidation of βHB yields more energy versus the CHO derived substrates: glucose, pyruvate and lactate [17, 70] (see Figure 1.3). Additionally, βHB oxidation exerts opposite redox effects on the NAD^+/NADH and co-enzyme Q couples, thus increasing the free energy liberated by ATP hydrolysis [69]. These effects may explain the 25% improvement in hydraulic efficiency (J·mol O_2^{-1}) in the working rat heart perfused with a physiological ratio of ketones and glucose versus glucose alone [69]. Whilst fat is more reduced than glucose, pyruvate, lactate or βHB (that is, fat contains more potential energy), its P/O ratio is higher, reflecting an increased oxygen cost per ATP synthesised [17, 26, 70]. Moreover, fat oxidation rates are low at intensities relevant to most competitive endurance events [18, 43]. Taken together, ketone oxidation in exercising skeletal muscle, particularly at exercise intensities typically reliant on CHO oxidation, may result in greater power output for a given volume of oxygen consumed [70] (that

is, improved exercise efficiency [73]). Whilst evidence for this hypothesis is lacking [71, 112, 122, 123], it is notable that VO_2 tended to be lower in the ketosis versus control condition in Chapter 3 (see Figure 3.4 D).

Hypothesised improvements in exercise efficiency during an exogenously-induced ketosis presumably rely on βHB oxidation contributing meaningfully to overall energy expenditure (although what constitutes a meaningful contribution is unclear). Yet, exogenous βHB oxidation rates during exercise have not been determined. To date, predictions are $\approx$0.35 g·min^{-1} ($\approx$18% of total oxygen consumption) during 40% W_{Max} exercise and $\approx$0.5 g·min^{-1} ($\approx$16% of total oxygen consumption) during 75% W_{Max} exercise [71]. However, these estimates relied on adjusted indirect calorimetry measurements [20], which are not validated for the unique physiological state of an exogenously-induced ketosis. As such, direct measures in exercising humans are warranted. Furthermore, factors affecting βHB oxidation during exercise are not well investigated. In particular, it is unclear how exercise intensity and blood βHB concentration affect exogenous βHB oxidation rates. Notably, in Chapter 3, during incremental-intensity exercise, blood βHB concentration fell significantly at low to moderate exercise intensities (100 W to 200 W) but thereafter plateaued (Figure 3.2), suggesting that βHB oxidation may have been reduced at higher workloads.

Therefore, the primary aims of this study were to determine: (i) the direct contribution of exogenous βHB oxidation to overall energy expenditure during exercise using a βHB stable isotope tracer; (ii) the effect of altering blood βHB concentration on exogenous βHB oxidation rates during exercise; and (iii) the effect of exercise intensity on exogenous βHB oxidation rates. Additionally, this work sought to determine whether supplementing athletes with βHB enhanced exercise efficiency.

4.2 Methods

4.2.1 Subjects and recruitment

Six healthy, athletically trained subjects participated in this single-blinded, cross-over-design (three conditions), random-order-controlled study. Subjects were recruited from advertisements at local sports clubs or via direct invitation to participate. Participant characteristics are presented in Table 4.1. Subjects were actively participating in either cycling, running, triathlon or swimming. Inclusion and exclusion criteria matched those presented in paragraph 2.2.

Table 4.1: **Participant characteristics**

	Mean (SD)
Gender (m/f)	5/1
Age (yr)	31.3 (3.9)
Height (m)	1.86 (0.14)
Weight (kg)	78.7 (14.3)
BMI ($kg \cdot m^{2\text{-}1}$)	22.6 (1.0)
Training $h \cdot wk^{-1}$	15.7 (6.1)
VO_2 peak ($L \cdot min^{-1}$)	4.5 (0.8)
Weight adjusted VO_2 peak ($mL \cdot kg^{-1}$)	56.7 (4.3)
W_{Max} (W)	390.3 (68.6)

4.2.2 Baseline exercise test

Between one and two weeks before the first glycogen depletion visit, participants attended DPAG to undertake a baseline exercise test on a bicycle ergometer (see paragraph 2.4.1 for test details and Figure 4.1 for an illustration of the study protocol). The W_{Max} achieved was used to determine exercise intensities for subsequent study visits. These tests also served as familiarisation sessions to the laboratory and the equipment setup.

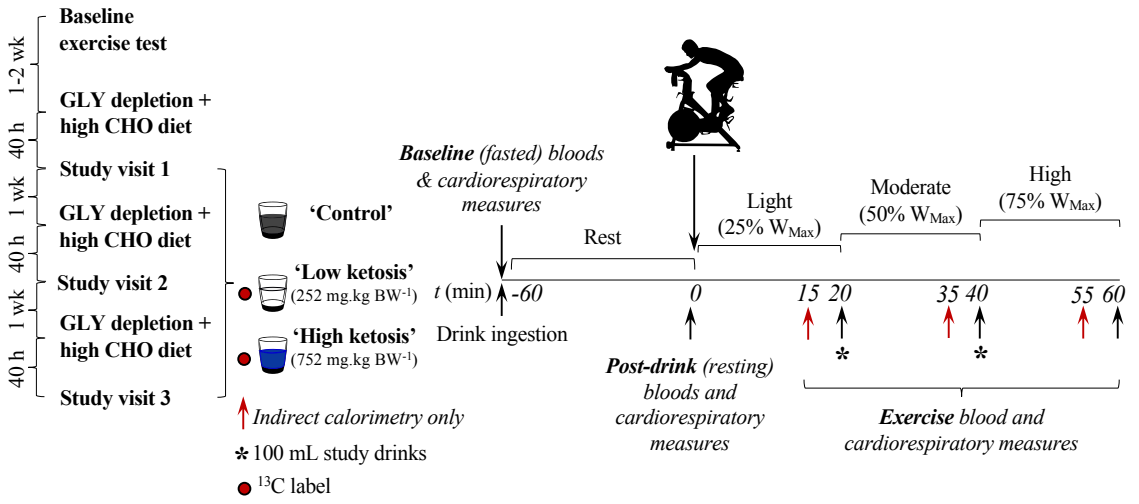


Figure 4.1: **Study visit protocol.** Participants (n=6) attended the laboratory on three occasions to undertake a standardised exercise protocol in a cross-over, random-order-controlled study. Before exercise, participants consumed either a control (bitter flavoured water), low dose ketone monoester drink (low ketosis) or a high dose ketone monoester drink (high ketosis). Ketone monoester drinks contained a carbon 13 (^{13}C) tracer, allowing the determination of exogenous βHB oxidation rates from sampled respiratory gases.

4.2.3 Pre-study visit diet and exercise standardisation

Measures were taken to ensure participants had a consistent preparation before all study visits. Approximately 40 hours prior to each study visit, participants undertook a supervised glycogen depleting exercise protocol on the same bicycle ergometer (Ergoselect 100, Ergoline, Germany) followed by a high CHO content diet (≈70% of total energy intake) and no additional exercise (Figure 4.1). These visits sought to provide a consistent level of fatigue and similar intramuscular glycogen content for each study visit. The exercise protocol consisted of alternating 2 min "work" and "recovery" intervals commencing at 90% and 50% W_{Max}, respectively. When the participant could no longer hold 90% W_{Max} for 2 min (volitional fatigue or inability to hold a cadence ≥60 RPM), the work intensity was reduced by 10%. This continued until participants could no longer maintain 60% W_{Max} during work intervals. The number of completed repetitions were recorded. Participants were instructed to hold a cadence of 80 - 100 RPM and the bicycle ergometer was set to

the RPM-independent mode (meaning power was held constant, regardless of the cadence). This protocol reliably depletes intramuscular glycogen stores [107].

Following each glycogen depletion visit, participants were instructed to maintain a high CHO diet (target of 70% of total energy intake from CHO) and refrain from further exercise. Glycogen depleting exercise immediately followed by a CHO-rich diet is well-observed to result in glycogen supercompensation [33]. Participants were asked to avoid foods naturally abundant in ^{13}C (for example, corn sugars) and to refrain from alcohol. Participants kept a food diary between the glycogen depletion and study visits (≈40 hours) and were asked to closely match their food intake before each study visit.

4.2.4 Study visits

Each subject performed three exercise trials, separated by ≈1 week (Figure 4.1). Participants reported to the laboratory at ≈0800 having fasted overnight. Two baseline (t = -60 min; "pre-drink") arterialised venous blood samples were drawn (see paragraph 2.4.3 for a description of the sampling technique): (i) for the immediate analysis of blood gases (≈1 mL); and (ii) for the analysis of blood substrate and insulin concentrations (≈3 mL). The second sample was stored in an ice-water bath for the duration of the visit. Cardiorespiratory measures were made via indirect calorimetry (see paragraph 2.4.2) and participants scored their resting exertion (scales described below) and gastrointestinal discomfort (scales described in [119]). Finally, respiratory gas samples were collected via a Douglas bag (see paragraph 2.4.2).

Following completion of baseline measurements, participants consumed a 400 mL bolus study drink, either: (i) a calorie-free control drink ("control", bitter flavoured water); (ii) a "low" dose ketone monoester drink ("low ketosis"; target blood βHB concentration of 2 - 3 mM); or (iii) a "high" dose ketone monoester drink ("high ketosis"; target blood βHB concentration of >4 mM; Figure 4.1). The order participants completed the three study conditions was randomly controlled and participants were

blinded to the condition they were undertaking. Participants then rested for 60 min. Following consumption of study drinks, subjects were asked to collect all urine in a provided container.

At $t = 0$ min ("post-drink"), all baseline measurements (as described above) were repeated. Participants then undertook 60 min of continuous cycling ergometer exercise, comprising of 20 min intervals at 25% W_{Max} ($t = 0$ – 20 min), 50% W_{Max} ($t = 20$ – 40 min) and 75% W_{Max} ($t = 40$ – 60 min) exercise intensities. Participants were instructed to maintain a cadence of 80 - 100 RPM and the bicycle ergometer was set to the RPM-independent mode. Room temperature and airflow were consistent across study visits. Participants consumed water ad libitum. During exercise, cardiorespiratory, RPE and gastrointestinal discomfort measurements were made at $t = 15$, 20, 35, 40, 55 and 60 min. Breath and blood samples (x2) were collected at $t = 20$, 40 and 60 min. To ensure blood βHB concentrations remained stable throughout exercise, 100 mL study drinks were administered at the end of the 25% W_{Max} and 50% W_{Max} exercise intervals.

4.2.5 Drink preparations

A 600 mL study drink was prepared containing 252 mg·kg BW^{-1} ketone monoester for low ketosis and 752 mg·kg BW^{-1} ketone monoester for high ketosis conditions. Thus, based on a mean participant weight of 79 ± 4 kg, the mean amount of βHB delivered was 19.9 ± 1.0 g (95.5 ± 4.8 kcal) in the low ketosis condition and 59.4 ± 3.0 g (285.1 ± 14.4 kcal) in the high ketosis condition. Control study drinks were calorie-free, isovolumetric amounts of bitter flavored (product code: 648352, Symrise, Germany) water. All drinks also included a sweetener (Neotame NutraSweet, Manus Bio, USA) to aid palatability. The dose response of ketone monoester drinks was predicted from previous work investigating its pharmacokinetics [116]. The 600 mL study drinks were divided into a 400 mL bolus consumed before exercise ($t = 0$ min) and two 100 mL drinks, consumed at $t = 20$ and 40 min.

To determine exogenous βHB oxidation rates, a ^{13}C labelled ketone monoester

was created and incorporated into study drinks. Ninety-nine percent U-^{13}C-labelled ethyl-acetoacetate was purchased from Cambridge isotope laboratories (USA). Ethyl-acetoacetate was used as the precursor for the synthesis of both U-^{13}C R-1,3-β-hydroxybutyrate and U-^{13}C 1,3-butanediol in separate fractions. Both fractions underwent a lipase reaction to produce 50% U-^{13}C-labelled ketone monoester with labels present in both the 1,3-β-hydroxybutyrate and U-^{13}C 1,3-butanediol "halves" of the final monoester (see Appendix F for a detailed description of βHB stable isotope preparation and synthesis). All synthesis was performed under good manufacturing practice conditions. Small aliquots of the ^{13}C-containing ester were incorporated into unlabeled ketone monoester drinks, resulting in a relative enrichment of ≈25%.

4.2.6 Blood gas analysis

Whole blood samples (≈1 mL) were immediately analysed using a benchtop blood gas analyser (ABL800, Radiometer UK, UK). Attained pH and HCO_3^- values were subsequently corrected using a custom MATLAB script (v2017a, MathWorks, USA), which accounted for the increased Hb saturation in arterialised blood (see Appendix C for a description of the calculations used).

4.2.7 Blood substrate analysis

βHB was analysed immediately from whole blood samples using a portable analyser (Freestyle Optium Neo, Abbott Laboratories, USA). Following completion of the visit, samples kept on ice were centrifuged for 10 min at 3600 RPM. The serum component was aliquoted into Eppendorf tubes and these were stored at -80 °C until later analysis. Frozen samples were thawed at room temperature for ≈30 min before biochemical analyses were undertaken. Glucose, NEFA and lactate were assayed in serum samples using a calibrated, semi-automated benchtop analyser (Pentra C400, Horiba Medical, France). All analyses were performed in duplicate, with the average value taken.

4.2.8 Urine sampling and analysis

The total volume of urine collected during each study visit was recorded. A 1 mL sample was taken and stored at -80 °C until later analysis. Urine βHB was assayed in thawed samples using a semi-automated benchtop analyser (Pentra C400, Horiba Medical, France).

4.2.9 Determination of exogenous βHB oxidation rates

The ^{13}C enrichment in ketone monoester drinks was determined by gas chromatography-mass spectrometry after derivatisation with a trimethylsilyl group [215]. Expired CO_2 enrichment was established through gas chromatography-combustion-isotope ratio mass spectrometry, using previously published methods [216]. Exogenous βHB oxidation rate (g·min^{-1}) was calculated following determination of the tracer to tracee ratio (TTR) (carbon 13 labelled CO_2 ($^{13}CO_2$):carbon 12 labelled CO_2 ($^{12}CO_2$) in respiratory gas samples and ^{13}C:carbon 12 (^{12}C) in drink samples), as described in equation (4.1) [215].

$$VCO_2.((TTR\ CO_{2\ exp} - TTR\ CO_{2\ bkg})/(TTR_{\ KME} - TTR_{\ bkg\ drink})).(1/k) \quad (4.1)$$

Calculations assumed that: 1 mol of gas = 22.4 L under standard temperature and pressure conditions; the molar mass of βHB is 104.1 g·mol^{-1}, and the volume of CO_2 produced by the oxidation of βHB (k) is 0.86 L. Abbreviations in equation 4.1 not previously defined include: KME = ketone monoester, bkg = background enrichment and exp = expired. The background drink represented water with sweeteners.

4.2.10 Calculations of whole-body efficiency

Energy expenditure (kcal.min^{-1}) was calculated according to the exercise intensity-dependent formulas proposed by Jeukendrup and Wallis [217]:

$$\begin{aligned} 25\%\ and\ 50\%\ W_{Max} &= (0.575.VCO_2) + (4.435.VO_2) \\ 75\%\ W_{Max} &= (0.550.VCO_2) + (4.471.VO_2) \end{aligned} \tag{4.2}$$

The delta efficiency, which represents the change in energy expenditure relative to the change in work accomplished [73], was determined by calculating the individual linear regression (y = mx + b) of the relationship between energy expended.min^{-1} versus work accomplished.min^{-1} [218]. Delta efficiency was then calculated from the reciprocal of the slope of this relationship (1/m) and expressed as a percentage. Delta efficiency is thought to provide a more valid reflection of changes in muscular efficiency during exercise versus other commonly used measures, such as gross efficiency [73].

4.2.11 Ratings of perceived exertion

Subjective scores of RPE ("What is your perceived exertion?"), breathlessness ("How breathless are you?"), leg discomfort ("What is your leg discomfort?"), anxiety of breathing ("How anxious are you about your breathing?") and anxiety of leg discomfort ("How anxious are you about your leg discomfort?") were recorded in a random order on a linear scale of 0 – 10 (0 = none to 10 = maximum).

4.2.12 Skeletal muscle biopsy and phenotyping

The activity of BDH and SCOT (which catalyse the first and second reactions of ketolysis, respectively) is markedly greater in type I (slow-oxidative) versus types IIa and IIb skeletal muscle fibres [60]. As such, an individual's muscle phenotype (relative proportion of each fibre) may influence exogenous βHB oxidation rate. Therefore, to give context to exogenous βHB oxidation data, skeletal muscle fibre phenotyping was undertaken.

A skeletal muscle biopsy of vastus lateralis was performed on participants (see paragraph 2.1 for details of the procedure) prior to baseline measurements at the

control visit. Samples were subsequently sectioned, stained with an anti-slow skeletal myosin heavy chain antibody, imaged using a widefield fluorescence imaging system and analysed in ImageJ (NIH, USA) to determine the proportion of slow-oxidative muscle fibres for each participant (see paragraph 2.5.1 for full methods).

4.3 Statistics

All data reported are mean ± SD and statistical significance was taken as $p<0.05$. The statistical tests employed are reported in the Results section.

4.4 Results

4.4.1 Pre-study visit preparation

No order effects were observed for exercise capacity (number of steps completed) in the glycogen depleting exercise (data not shown). Neither the macronutrient composition nor total energy intake for the ≈40 hours preceding study visits differed between study conditions (Table 4.2). As requested, participants consumed a high CHO content diet, representing 61.09 ± 10.44%, 64.16 ± 8.42% and 57.09 ± 8.33% of total energy intake leading into the control, low ketosis and high ketosis conditions, respectively.

4.4.2 Blood βHB concentration

There were main effects of condition and time, and a condition-time interaction effect for blood βHB concentration (all $p<0.001$), which diverged in a dose-dependent manner following ingestion of the study drinks (Table 4.3). βHB concentration was increased by ≈2-fold in the high ketosis (mean ± SD of all post-drink values, 4.40 ± 0.35 mM) versus low ketosis (1.96 ± 0.31 mM) conditions at rest and throughout exercise. Blood βHB concentrations were unaffected by ingestion of the control drink. The consumption of 100 mL study drinks at the end of the 25% W_{Max} (20

Table 4.2: **Energy and macronutrient intake preceding study visits.** Data are taken from self-reported diet diaries. Statistical comparisons were made by 1-way ANOVA. Abbreviations not previously defined: TE = total energy.

	CHO		**Fat**		**Protein**		**TE**
	g·kg BW^{-1}	% TE	g·kg BW^{-1}	% TE	g·kg BW^{-1}	% TE	kcal.kg BW^{-1}
Control	11.34 ± 4.04	61.09 ± 10.44	2.02 ± 1.21	24.56 ± 11.22	2.79 ± 1.33	14.35 ± 2.94	74.67 ± 22.79
Low ketosis	10.84 ± 3.64	64.16 ± 8.42	1.85 ± 0.91	22.94 ± 10.47	2.24 ± 0.98	12.91 ± 2.70	68.95 ± 24.65
High ketosis	11.80 ± 6.23	57.09 ± 8.33	2.55 ± 0.85	29.35 ± 6.38	2.88 ± 1.56	13.57 ± 3.16	81.68 ± 35.37

min) and 50% W_{Max} (40 min) exercise intervals (see study protocol in 4.1) meant that blood βHB concentrations remained stable during exercise in the low and high ketosis conditions.

4.4.3 Gastrointestinal discomfort

A main effect of condition was present for nausea (p=0.009), with post-hoc analyses showing a tendency for this to be greater in the high ketosis (2.2 ± 2.3; 0 - 10 scale) versus control condition (no reported nausea; p=0.053; data not shown). No differences were observed for other upper/lower abdominal or systemic gastrointestinal distresses (data not shown).

4.4.4 Effect of blood βHB concentration and exercise intensity on exogenous βHB oxidation rate

Neither a main effect of condition, nor a condition-time interaction effect were present for exogenous βHB oxidation rates (Table 4.3 and Figures 4.2 A and B). Exogenous βHB oxidation rate was significantly associated with oxygen consumption (R^2=0.402, p<0.001; Figure 4.2 C). This relationship remained significant when resting values were removed, although the variance explained was just ≈11% (R^2=0.111, p=0.048) (Figure 4.2 D).

A main effect of time was observed for exogenous βHB oxidation rates (p<0.001). In both the low and high ketosis conditions, oxidation rates rose significantly from rest to the 25% W_{Max} exercise interval (≈7-fold increase), and from 25% W_{Max} to 50% W_{Max} exercise intervals (≈1.5-fold increase), but thereafter plateaued (Table 4.3). Peak exogenous βHB oxidation rates were 0.103 ± 0.044 g·min^{-1}, observed during the 75% W_{Max} exercise interval. In both conditions, the relative contribution to energy expenditure during exercise was highest during the 25% W_{Max} (4.46 ± 2.71%) and lowest during the 75% W_{Max} exercise intensities (2.48 ± 1.66%; Table 4.3).

Table 4.3: **βHB blood concentration, oxidation rates and urinary excretion.** Relative contributions to overall energy expenditure are given in parentheses. Statistical comparisons of blood βHB concentrations and exogenous βHB oxidation rates were made by 2-way RM ANOVA. Differences in urine βHB concentrations were investigated by 1-way RM ANOVA. Significant post-hoc comparisons: Ψ = versus control at equivalent measurement; # = versus low ketosis at equivalent measurement; * = versus post-drink (within group); § = versus 25% W_{Max} (within group); & = versus 50% W_{Max} (within group).

	Pre-drink	**Post-drink**		**25% W_{Max}**		**50% W_{Max}**		**75% W_{Max}**		
	βHB (mM)	βHB (mM)	βHB ox. (g·min^{-1})	βHB (mM)	βHB ox. (g·min^{-1})	βHB (mM)	βHB ox. (g·min^{-1})	βHB (mM)	βHB ox. (g·min^{-1})	Urine βHB (g)
Control	0.18 ± 0.04	0.20 ± 0.00		0.18 ± 0.04		0.17 ± 0.05		0.17 ± 0.05		0.001 ± 0.000
Low ketosis	0.20 ± 0.06	2.42 ± 0.53$^{\Psi}$	0.009 ± 0.003 (2.47 ± 1.52)	1.83 ± 0.48$^{\Psi}$	0.062 ± 0.031* (3.83 ± 2.12)	1.73 ± 0.65$^{\Psi}$	0.087 ± 0.053*§ (3.33 ± 2.01)	1.87 ± 0.73	0.098 ± 0.068 (2.48 ± 1.66§)	0.060 ± 0.034$^{\Psi}$
High ketosis	0.17 ± 0.05	4.90 ± 1.03$^{\Psi\#}$	0.012 ± 0.011 (3.37 ± 3.48)	4.25 ± 1.65$^{\Psi\#}$	0.079 ± 0.054* (4.46 ± 2.71)	4.10 ± 1.38$^{\Psi\#}$	0.100 ± 0.058*§ (3.56 ± 1.68)	4.33 ± 1.05	0.103 ± 0.044 (2.58 ± 1.05)	0.318 ± 0.188$^{\Psi}$

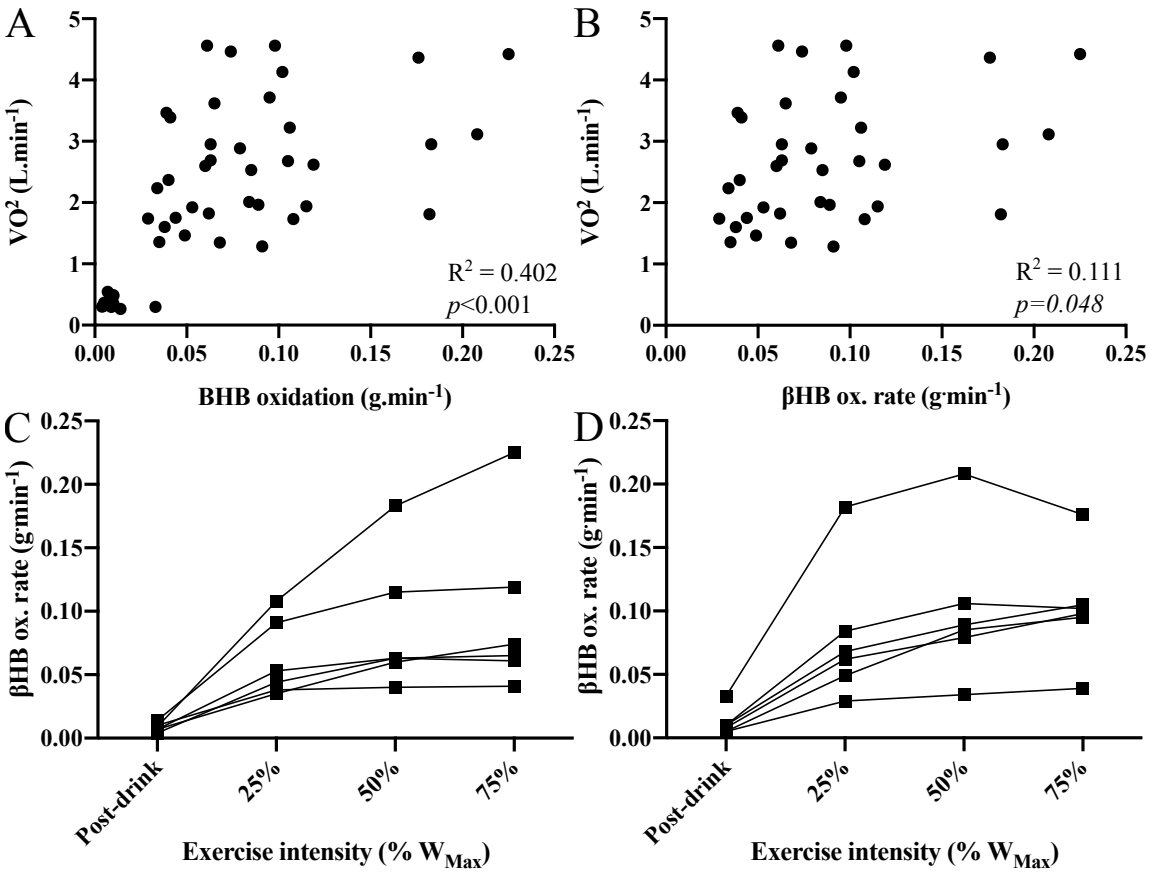


Figure 4.2: **Individual exogenous βHB oxidation rates and associations between exogenous βHB oxidation rate and the volume of oxygen consumed.** Association between exogenous βHB oxidation rate and VO_2 **(A)** with resting (post-drink) values and **(B)** during exercise only (determined through simple linear regressions). Individual exogenous βHB oxidation rates during the **(C)** low ketosis and **(D)** high ketosis conditions.

4.4.5 Urine βHB concentration

A main effect of condition was present for urine βHB concentration (p<0.001), which was greater in low ketosis versus control condition (p=0.002) and in the high versus low ketosis condition (p=0.004) (Table 4.3).

4.4.6 Cardiorespiratory measures and ratings of perceived exertion

As expected, there was a main effect of time for all measured cardiorespiratory and RPE values (all p<0.001), which rose in accordance with exercise intensity

(Table 4.4, and Appendix G for components of RPE and anxiety related to these). However, no main effects of condition, nor condition-time interaction effects were present. Importantly, no within-condition differences were observed for VO_2, V_E, HR or RPE measures taken within the last 5 min of each exercise interval (for example, t = 15 min versus t = 20 min for the light intensity interval) indicating that athletes had achieved a physiological "steady state" at the point of respiratory gas collection for determination of exogenous βHB oxidation rates.

4.4.7 Respiratory exchange ratio and exercise efficiency

There was a main effect of time (p<0.001) and tended to be a main effect of condition for RER (p=0.051; Figure 4.3 A). However, a time-condition interaction effect was not present. Exploratory post-hoc comparisons of main condition effects showed that RER tended to be lesser in the low ketosis versus control condition (p=0.051). Delta efficiency also tended to be different between conditions (ANOVA, p=0.098; Figure 4.3 B). Exploratory post-hoc analyses showed that delta efficiency was significantly greater in the low ketosis versus control condition (25.94 ± 2.11% versus 24.09 ± 1.91%, respectively; p=0.027).

4.4.8 Blood substrate concentrations

There was a main effect of time (p=0.003) and condition-time interaction (p=0.031) effect for blood glucose concentration, which was significantly lower in the high ketosis versus control condition at rest and following 25% W_{Max} exercise (p=0.036 and p=0.035, respectively) (Figure 4.4 A). Significant condition, time and condition-time interaction effects (all p<0.001) were present for blood NEFA concentration, which fell from ≈0.6 mM to ≈0.2 mM following ingestion of the ketone monoester drinks and was significantly lower at rest (post-drink) and throughout exercise in the low and high ketosis versus the control conditions (Figure 4.4 B). There were no differences in blood NEFA concentrations between low and high ketosis conditions at any time point. There was a main effect of time for blood lactate concentration

Table 4.4: **Cardiorespiratory measures and perceived exertion.** Exercise measurements are presented as time points with the relative workload in parentheses. Statistical comparisons of cardiorespiratory measures and RPE were made by 2-way RM ANOVA.

	Measurement	Control	Low ketosis	High ketosis
VO_2 ($L \cdot min^{-1}$)	Pre-drink (rest)	0.31 ± 0.08	0.28 ± 0.07	0.31 ± 0.03
	Post-drink (rest)	0.30 ± 0.06	0.38 ± 0.11	0.37 ± 0.07
	15 min (25%)	1.62 ± 0.25	1.68 ± 0.35	1.64 ± 0.29
	20 min (25%)	1.55 ± 0.20	1.61 ± 0.25	1.70 ± 0.25
	35 min (50%)	2.60 ± 0.47	2.60 ± 0.46	2.64 ± 0.55
	40 min (50%)	2.65 ± 0.45	2.58 ± 0.39	2.66 ± 0.50
	55 min (75%)	3.78 ± 0.86	3.63 ± 0.80	3.75 ± 0.83
	60 min (75%)	3.89 ± 0.87	3.85 ± 0.77	3.82 ± 0.69
VCO_2 ($L \cdot min^{-1}$)	Pre-drink (rest)	0.24 ± 0.08	0.22 ± 0.07	0.24 ± 0.04
	Post-drink (rest)	0.25 ± 0.06	0.28 ± 0.29	0.29 ± 0.05
	15 min (25%)	1.35 ± 0.28	1.37 ± 0.31	1.32 ± 0.32
	20 min (25%)	1.27 ± 0.23	1.30 ± 0.22	1.38 ± 0.27
	35 min (50%)	2.22 ± 0.40	2.20 ± 0.45	2.23 ± 0.51
	40 min (50%)	2.28 ± 0.38	2.16 ± 0.41	2.21 ± 0.49
	55 min (75%)	3.52 ± 0.85	3.34 ± 0.84	3.35 ± 0.86
	60 min (75%)	3.67 ± 0.78	3.48 ± 0.82	3.44 ± 0.72
V_E ($L \cdot min^{-1}$)	Pre-drink (rest)	10.28 ± 1.58	8.76 ± 3.11	9.27 ± 2.11
	Post-drink (rest)	10.78 ± 2.17	11.34 ± 3.46	12.99 ± 1.91
	15 min (25%)	41.13 ± 5.02	42.05 ± 6.86	45.13 ± 9.40
	20 min (25%)	38.37 ± 5.30	41.09 ± 4.03	46.46 ± 8.77
	35 min (50%)	63.74 ± 8.39	64.94 ± 9.13	70.76 ± 11.27
	40 min (50%)	65.52 ± 5.43	64.57 ± 8.37	68.34 ± 19.56
	55 min (75%)	104.59 ± 20.35	103.22 ± 22.06	105.27 ± 19.56
	60 min (75%)	107.84 ± 17.11	105.77 ± 19.31	112.34 ± 19.74
HR (bpm)	Pre-drink (rest)	47.33 ± 6.38	48.67 ± 7.26	47.33 ± 7.03
	Post-drink (rest)	51.33 ± 5.65	54.50 ± 7.45	57.67 ± 10.05
	15 min (25%)	97.40 ± 7.06	102.40 ± 8.41	104.40 ± 4.93
	20 min (25%)	98.33 ± 5.01	97.83 ± 12.04	104.17 ± 7.86
	35 min (50%)	125.83 ± 10.50	124.50 ± 13.49	128.17 ± 9.15
	40 min (50%)	125.83 ± 10.52	127.50 ± 10.29	127.83 ± 10.30
	55 min (75%)	156.83 ± 9.04	153.67 ± 11.78	153.00 ± 9.17
	60 min (75%)	158.00 ± 8.79	156.33 ± 12.72	154.17 ± 8.38
RPE (0 - 10)	Pre-drink (rest)			
	Post-drink (rest)			
	15 min (25%)	1.00 ± 0.82	1.50 ± 0.58	0.88 ± 0.25
	20 min (25%)	0.92 ± 0.66	1.42 ± 0.66	0.83 ± 0.26
	35 min (50%)	2.63 ± 0.75	3.25 ± 0.96	3.25 ± 0.87
	40 min (50%)	2.70 ± 0.84	2.60 ± 1.14	2.60 ± 1.08
	55 min (75%)	6.63 ± 0.48	6.75 ± 1.26	6.25 ± 0.87
	60 min (75%)	6.33 ± 1.37	6.00 ± 2.00	5.58 ± 1.91

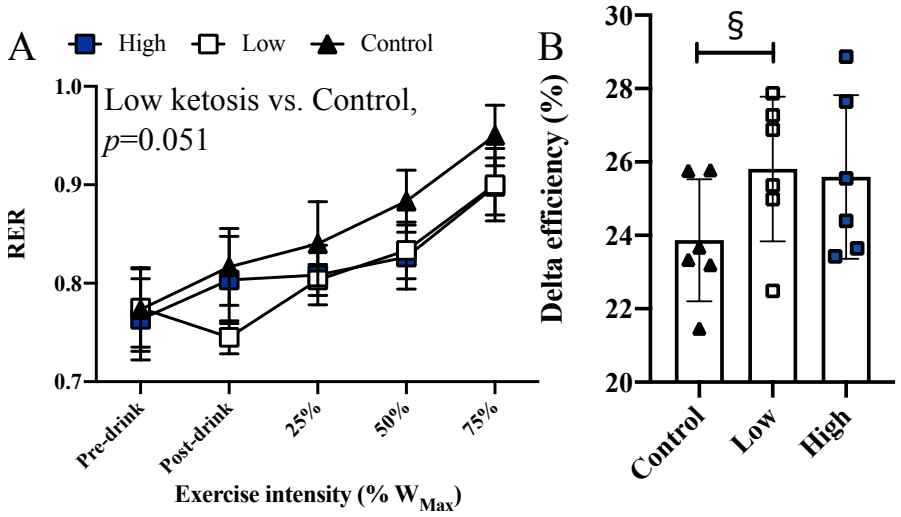


Figure 4.3: **Respiratory exchange ratio and exercise efficiency. (A)** RER at rest and during exercise. **(B)** Delta efficiency (%) representing the relationship between energy expended.min^{-1} versus work accomplished.min^{-1} during exercise. Differences in RER and delta efficiency were investigated by 2-way RM ANOVA and 1-way RM ANOVA, respectively. Significant post-hoc comparisons: § = between control and low ketosis conditions.

(p<0.001) but no condition or condition-time interaction effects (Figure 4.4 C).

4.4.9 Blood acid-base balance

There were significant main effects of condition and time (both p<0.001) for blood pH, which was reduced in the low ketosis versus control condition (p=0.002) and high versus low ketosis condition (p<0.001; Figure 4.4 D). A significant negative correlation was present between blood βHB concentration and pH in the ketosis conditions (R^2=0.455, p<0.001; Figure 4.4 E). In the high ketosis condition, participants were mildly acidotic (pH 7.33 ± 0.03) prior to commencing exercise. As would be expected, a similar pattern was observed for blood HCO_3^- where main effects of condition (p=0.004) and time (p<0.001), and a condition-time interaction (p<0.001) effect were present. Blood HCO_3^- was lower in the high and low ketosis conditions versus control at rest (post-drink) and throughout exercise (Figure 4.4 F).

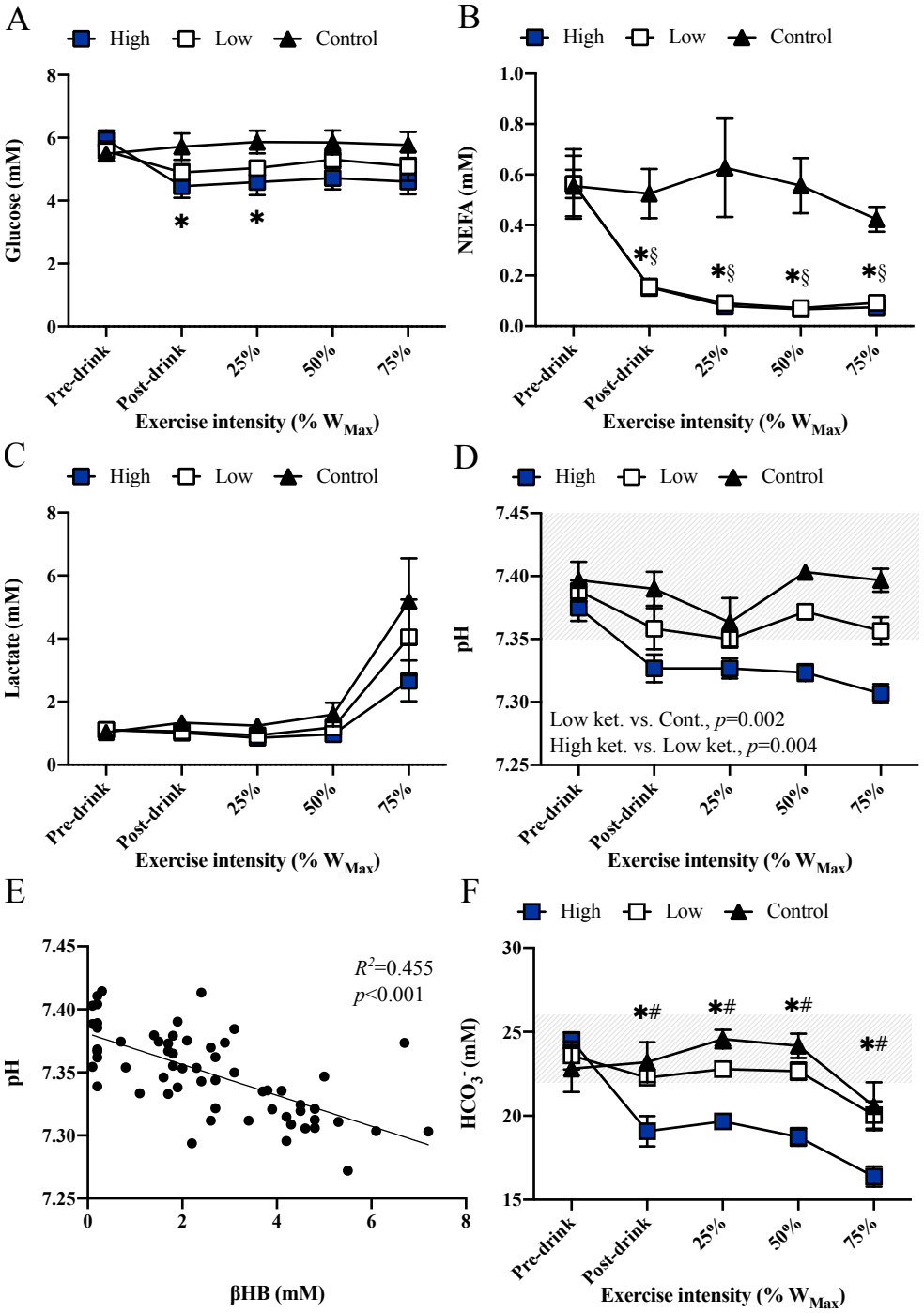


Figure 4.4: **Blood substrates and blood gases.** Blood **(A)** glucose; **(B)** NEFA; and **(C)** lactate concentrations at rest and during exercise. **(D)** Blood pH at rest and during exercise. Shaded area represents the normal blood pH range. **(E)** Relationship between blood βHB concentration and pH in the low and high ketosis conditions. **(F)** Blood HCO_3^- concentration at rest and during exercise. Shaded area represents the normal blood HCO_3^- range. Blood substrate and blood gas data were analysed by 2-way RM ANOVA. Significant post-hoc comparisons: * = between control and high ketosis conditions; § = between control and low ketosis conditions; # = between low and high ketosis conditions.

4.4.10 Association between muscle fibre type and βHB oxidation rate

There was a significant positive association between the percentage of slow-oxidative muscle fibres in vastus lateralis muscle biopsy samples and peak exogenous βHB oxidation rate (R^2=0.696, p=0.039; Figure 4.5 and Appendix H, which presents example images of muscle staining for each participant).

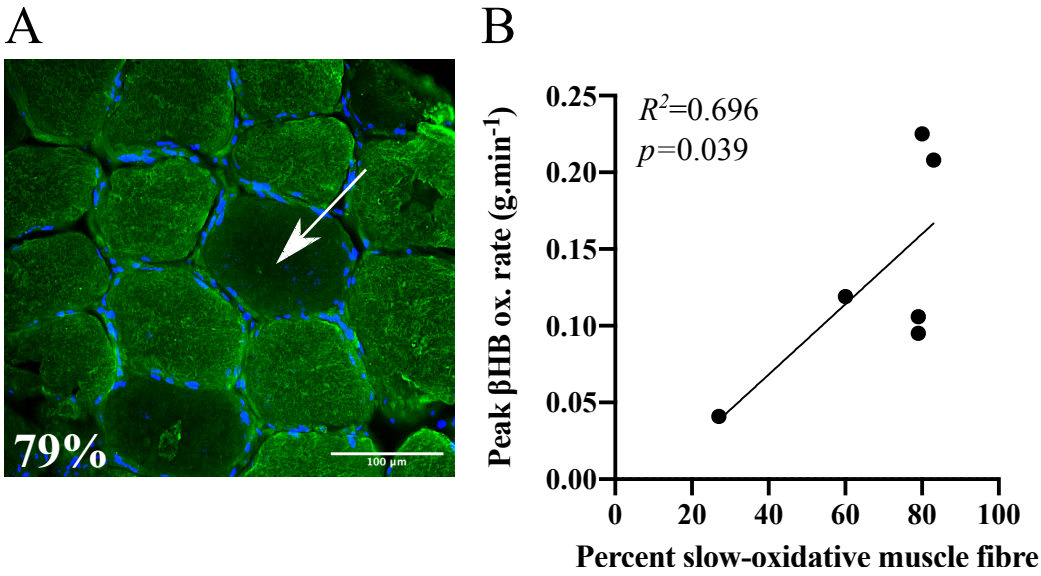


Figure 4.5: **Association between skeletal muscle fibre type and peak exogenous βHB oxidation rate. (A)** Vastus lateralis biopsy sample stained with an anti-slow skeletal myosin heavy chain primary antibody (fluorescent green). Nuclei are represented in blue (stained with DAPI). An example non-stained fibre (representing fast-glycolytic (IIa) and fast (IIb) fibres) is indicated with the white arrow. The percentage value represents the proportion of slow-oxidative fibres (minimum fibre count of 50). **(B)** Relationship between peak exogenous βHB oxidation rate (g·min^{-1}) and skeletal muscle phenotype (determined through simple linear regression).

4.5 Discussion

The main findings of this study were that: (i) increasing blood βHB concentration from ≈2 mM to ≈4.4 mM did not affect exogenous βHB oxidation rates at rest or during incremental intensity exercise; (ii) the relative contribution of exogenous βHB oxidation to energy expenditure during exercise was low (2.48% to 4.46%) and

peaked during the 25% W_{Max} exercise intensity; (iii) exercise efficiency (measured as delta efficiency) was improved when blood βHB concentration was raised to ≈2 mM.

4.5.1 Exogenous βHB oxidation contributes minimally to energy expenditure during exercise

The peak relative contribution of exogenous βHB oxidation to energy expenditure was 4.46 ± 2.71%. This is low compared to values reported for FFAs (31% at 40% W_{Max}), other fat sources (27% at 40% W_{Max}), blood glucose (18% at 75% W_{Max}) and muscle glycogen (58% at 75% W_{Max}) during incremental-intensity cycling ergometer exercise [18]. Thus, exogenous βHB appears to contribute minimally to whole-body energy expenditure during exercise.

Previous work predicted between 16% and 18% of oxygen consumption was attributable to βHB oxidation when similarly highly-trained athletes were given a drink containing CHO and 573 mg·kg BW^{-1} ketone monoester prior to cycling ergometer exercise [71]. In the present study, assuming the complete oxidation of βHB requires 4.5 mol of O_2 and that 1 mol of gas occupies 22.4 L [20], the peak contribution of exogenous βHB oxidation to VO_2 was ≈4.5%. Without the capacity to directly trace whole-body exogenous βHB oxidation (as performed in this study), previous estimations utilised adjusted substrate oxidation equations from measured gaseous exchanges [71]. Inherent to these equations is the assumption that βHB R_d from the blood (difference in blood βHB area under the curve (AUC) (mmol·L^{-1}.min^{-1}) at rest and during exercise) would accurately reflect exogenous βHB oxidation. However, a substantial proportion of the blood βHB R_d may have been attributable to intermediate metabolic processes; notably, the conversion of βHB to AcAc and its subsequent spontaneous decarboxylation to acetone. Assuming AcAc was subsequently oxidised, or that any acetone produced was excreted in respiratory gases, these intermediate metabolic processes would not affect overall conclusions regarding substrate metabolism [20]. However, when there is an accumu-

lation within non-blood body compartments, or non-respiratory gas excretion from the body of these metabolites (discussed further below), respiratory gas equations would overestimate exogenous βHB oxidation rates. This may account for some of the discrepancy between findings here and those previously reported [71].

Whole-body oxidation accounted for ≈27.5% (5.5 g) and ≈10.7% (6.4 g) of βHB consumed in the low and high ketosis conditions, respectively. Assuming a βHB volume of distribution of 0.2 L·kg BW^{-1} [215] (although values as high as 0.5 L·kg BW^{-1} have been reported [219]), at the end of exercise (t = 60 min) the amount of βHB within the body fluids accounted for a further ≈15.5% (≈3.1 g) and ≈12.0% (≈7.1 g) of the βHB consumed in the low and high ketosis conditions, respectively. As in previous reports [71, 92], the urinary excretion of βHB was minimal (<1% in both conditions). Given the inhibitory effects of high circulating ketone concentrations on hepatic ketogenesis [48], it is assumed that βHB measured in urine and blood was almost entirely derived from the ketone monoester drinks. Thus, ≈57% of βHB in the low dose ketone monoester (252 mg·kg BW^{-1}) and 77% of βHB in the high dose ketone monoester (752 mg·kg BW^{-1}) remained unaccounted for.

Within the mitochondria, βHB is dehydrogenated to form AcAc in a near equilibrium reaction [48]. Previous work has demonstrated an ≈4:1 ratio of blood βHB to AcAc in healthy, overnight fasted subjects following ingestion of a 395 mg·kg BW^{-1} dose of the ketone monoester [92], consistent with that observed during an endogenously induced physiological ketosis [48]. Additionally, breath acetone, formed from the spontaneous decarboxylation of AcAc, was measured at 25 ppm after 1 hour rest (≈7.5 mg·min^{-1} acetone expelled, assuming a resting VO_2 of 0.3 L·min^{-1}), peaking at ≈70 ppm (≈21 mg·min^{-1}) after 3 hours [92]. The slower rate of appearance (Ra) of acetone is likely attributable to sequestration in lipid stores, represented by an acetone volume of distribution of ≈0.6 - 0.8 L·kg^{-1} [20]. Taken together, these unmeasured ketones may account for a substantial proportion of the unaccounted exogenous βHB.

4.5.2 Doubling blood βHB concentration did not alter exogenous βHB oxidation rates

Exogenous βHB uptake in skeletal muscle is saturated at a relatively low circulating concentration in resting subjects (≈0.8 mM versus a physiological plateau of ≈7 mM) [65]. Inactive skeletal muscle has relatively low energy demand; however, upon commencement of exercise a large proportion of muscle may be activated (depending on the form of exercise undertaken), leading to a dramatic increase in blood supply to, and energy flux within, this tissue [64]. Accordingly, exercise appears to "override" resting saturation kinetics: skeletal muscle ketone oxidation is between ≈5-fold [71] and ≈11-fold [220] higher upon commencement of exercise, resulting in a similar uptake per kg of tissue as the brain [65]. This suggests that skeletal muscle ketone oxidation is not restricted by ketone transport and ketolytic capacity per se. Given this, in the present study it was postulated that exogenous βHB oxidation rates during exercise would be proportional to blood concentrations; that is, significantly greater in the high versus low ketosis conditions.

In agreement with previous work [71, 220], we found exercise stimulated an ≈6-fold increase in exogenous βHB oxidation in both low and high ketosis conditions. However, despite an approximate doubling of circulating βHB, oxidation rates were similar between conditions at rest and throughout all exercise intensities. Thus, we conclude that exercise but not circulating concentrations (at least $\geq \approx 2$ mM) can stimulate an increase in βHB oxidation in skeletal muscle. This result is consistent with our observation that exogenous βHB is a minimal contributor to energy expenditure during exercise. The primary purpose of ketogenesis is to provide a supplementary substrate to support cerebral metabolism when CHO stores are challenged [47, 55]. Skeletal muscle is metabolically flexible and can meet most of its energy needs from the oxidation of abundant lipid stores [18]. As such, it is logical that ketone oxidation in skeletal muscle is tightly regulated, thus preserving these respiratory fuels for the brain.

4.5.3 The greatest relative contribution of exogenous βHB oxidation is during light intensity exercise

It was previously reported that following ingestion of 573 mg·kg BW^{-1} ketone monoester, βHB AUC was significantly decreased at greater exercise intensities and subsequently, positively correlated with oxygen consumption [71]. In agreement with this work, a significant association between exogenous βHB oxidation rate and VO_2 was observed here. Notably, this relationship remained significantly non-zero even when resting values were excluded (although the variation in exogenous βHB oxidation explained was substantially lower, representing just ≈11%). However, contrary to this previous report [71], exogenous βHB oxidation was not increased when the exercise intensity was raised from 50% W_{Max} to 75% W_{Max}. As described, previous estimates of exogenous βHB oxidation relied on βHB R_d from the blood. It is possible that the inhibition of ketolysis downstream of the BDH reaction (see Figure 1.2 for an illustration of ketolysis) could be increased at higher exercise intensities, resulting in an accumulation of AcAc and, in turn, acetone. Inhibitory mechanisms may include a depletion of the succinyl-CoA pool, which is a substrate for the SCOT catalysed reaction, or product inhibition from increased acetyl-CoA (primarily derived from CHO oxidation) on the ACAT1 catalysed reaction. In such a case, changes in blood βHB R_d would overestimate βHB oxidation.

Arguably of more practical importance is the relative contribution of exogenous βHB oxidation to overall energy expenditure, which was low throughout exercise and declined with successive increments in exercise load. This observation is consistent with the increased importance of the ATP synthesised/oxygen consumed ratio (P/O ratio) at higher exercise intensities [26], which is greater for pyruvate versus βHB [17, 70].

4.5.4 Exercise efficiency is increased during ketosis

Elevating blood βHB concentrations to ≈2 mM resulted in an ≈7% improvement in exercise efficiency versus the control condition. This effect was driven by small, non-significant reductions in both VO_2 and VCO_2 at the 50% and 75% W_{Max} exercise intensities during ketosis, leading to an improvement in the ratio of the change of work accomplished.min^{-1} (which was fixed) and the change in energy expended.min^{-1} (that is, delta efficiency [73]). βHB is more chemically reduced than pyruvate and its combustion increases the electron transport redox span, thus creating a greater ΔG' ATP for the generation of ATP [69, 70]. In the working rat heart perfused with a physiological ratio of ketones and glucose versus glucose alone, these effects were reported to increase hydraulic efficiency (J·mol O_2^{-1}) by 25% [69]. Thus, a convenient explanation for the observed improvement in exercise efficiency is that exogenous βHB oxidation in exercising skeletal muscle resulted in lower oxygen consumption at the same (fixed) power output. However, it is unclear whether a peak ≈2.5% - 4.5% contribution from exogenous βHB oxidation to energy expenditure would be sufficient to drive the observed improvements in exercise efficiency.

It is plausible that improvements in exercise efficiency resulted from ketosis mediated alterations in CHO and fat metabolism. However, the oxidation of fat appears to be augmented during an exogenously-induced ketosis [71], which has a lower P/O ratio versus CHO (reflecting a reduction in the ATP produced versus oxygen consumed) [26] and, therefore, would be expected to lower exercise efficiency [101]. Further work is required to both confirm the observed improvement in exercise efficiency during an exogenously-induced ketosis and to determine the mechanisms contributing to this effect.

4.5.5 Skeletal muscle fibre phenotype may influence exogenous βHB oxidation rate

The activity of BDH and SCOT (which catalyse the first and second reactions of ketolysis) are ≈1.5 and ≈2.5 times greater in slow-oxidative versus fast glycolytic rat skeletal muscle fibres, respectively [60]. As such, it is postulated that the uptake and utilisation of ketones is greater in athletes with a slow-oxidative skeletal muscle phenotype [106]. The present work provides preliminary findings supporting this hypothesis. Participants' muscle phenotype explained ≈70% of variation in exogenous βHB oxidation rate. Although a plausible finding, these data should be interpreted with caution as the association was driven by one participant with an extremely fast muscle phenotype (former national-level sprinter, undertaking training for 5 - 10 km running at the time of enrolment). Notably, this individual had an ≈1.5-fold increase in blood βHB AUC and an ≈2-fold increase in blood lactate AUC versus the mean for the remaining participants. Furthermore, their blood pH and HCO_3^- was consistently lower during ketosis versus their counterparts. These results are coherent with a reduced uptake and oxidation of exogenous βHB in this athlete. Further work is warranted to test these preliminary results indicating that skeletal muscle phenotype is an important modulator of exogenous βHB oxidation in exercising humans.

4.5.6 Ketosis-mediated changes in circulating substrate concentrations was not affected by elevating circulating βHB concentrations

Previous work has shown sequential decreases in blood glucose and glycerol concentrations with successive increases in blood βHB concentrations of ≈0.5 mM, 0.8 mM and 1.7 mM [65]. Notably, the greatest decreases were observed from baseline (pre-infusion) to ≈0.5 mM [65]. In this study, no differences were observed for glucose, NEFA or lactate concentrations between blood βHB concentrations of ≈2 mM and

≈4.4 mM, either at rest or during exercise. Taken together, these results provide evidence for a blood βHB concentration threshold for ketosis-mediated alterations in circulating substrate concentrations, which is likely to occur ≤2 mM.

4.5.7 Acid-base balance is perturbed in a ketone monoester-dose-dependent fashion

Consumption of the ketone monoester drinks caused dose-dependent falls in blood pH (ketoacidaemia). Notably, participants in the high ketosis condition were acidotic prior to commencing exercise (pH = 7.32). Furthermore, whilst blood buffering capacity corrected towards control values in the low ketosis condition, HCO_3^- remained significantly depressed throughout exercise in the high ketosis condition. Unlike the work presented in Chapter 3 [121, 221], ketoacidaemia did not alter participants' perceptions of exercise exertion, nor their emotional responses to these perceptions, at any exercise intensity. Furthermore, these were unaffected by the degree of ketoacidaemia induced (low versus high ketosis). This may be due to the comparatively lower exercise intensities and longer exercise intervals used in this study, with even minor changes in the relationship between time and power output having been shown to yield differences in the magnitudes of reported exercise exertion [222]. Moreover, the rationale for including RPE measures within this study was to help ascertain (together with other cardiorespiratory measures) whether participants had achieved a "steady state" at the end of each exercise interval - not to determine the effects of blood βHB concentrations on subjective perceptions of exercise exertion, which given the design employed (three conditions, six time points) the study was underpowered to achieve.

4.5.8 Limitations and future directions

βHB oxidation results must be interpreted in the context of an exogenously-induced ketosis. It is very likely that whole-body oxidation rates during exercise would be different in the setting of a starvation or dietary induced ketosis. Participants

recruited had been consuming a "normal", CHO-rich diet prior to enrolment. Skeletal muscle is a highly plastic tissue, capable of altering its morphology and metabolism in response to a range of environmental stimuli, including diet [36]. As such, it is possible that exogenous βHB oxidation rates and relative contributions to overall energy expenditure would be greater in individuals chronically exposed to ketones ("keto-adapted" [99, 100]), for example through adoption of a ketogenic diet or even chronic consumption of exogenous ketones. While preliminary data demonstrated that skeletal muscle phenotype influences exogenous βHB oxidation rates, further work in a wider population of athletes is warranted to test this association.

4.6 Conclusion

Exogenous βHB oxidation contributes minimally to energy expenditure at rest and during exercise in highly trained athletes. Furthermore, oxidation rates are not affected by doubling blood βHB from ≈2 mM to ≈4.4 mM. Whilst peak exogenous βHB oxidation occurs at a light to moderate exercise intensity (50% W_{Max}), the greatest relative contribution is during light exercise (25% W_{Max}). Finally, despite low βHB oxidation rates, exercise efficiency is improved when blood βHB concentration is elevated to ≈2 mM.

Chapter 5

The role of ketones in the adaptive response to exercise training

5.1 Introduction

Skeletal muscle is a highly "plastic" tissue, being able to change its morphology and metabolism in response to environmental stimuli [36]. This plasticity is central to the adaptations achieved through exercise training [36]. Exercise causes transient increases in gene expression specific to the type, duration and intensity of physical activity performed [223]. Over time, this results in same-directional changes in intramuscular protein content that enhance fatigue resistance [38–40, 224, 225].

Other environmental factors can modulate adaptive changes made to exercise training [226, 227], including the composition of an athlete's diet [228, 229]. For example, adopting a ketogenic diet ($\approx \leq$10% of energy from CHO, see paragraph 1.2.5) may augment potentially advantageous molecular and phenotypic adaptations made in response to endurance exercise training [230]. These include further increasing the expression of enzymes/proteins that regulate fatty-acyl transport/catabolism and mitochondrial phosphorylation beyond that achieved through exercise training alone [43, 228, 231]. This contributes to markedly increased rates of fat oxidation during exercise, thus "freeing" athletes from a dependence on finite CHO stores [103]. Subsequently, it is postulated that the fat adaptation achieved from adoption of a ketogenic diet may enhance endurance exercise capacity [99]. However, evidence

for this is lacking [43]. Whilst adopting a ketogenic diet might be a useful training tool to augment potentially favourable adaptations to exercise training, exercise performed in a CHO-depleted state predisposes athletes to non-functional overreaching [232]. Furthermore, a ketogenic diet can induce insulin resistance in skeletal muscle and impair CHO metabolism [233, 234]. Subsequently, athletes' high-intensity exercise capacity is substantially blunted [100, 235].

In contrast, whether the unique physiological state of an exogenously-induced ketosis may affect metabolic adaptations to endurance exercise training has not been elucidated. Interestingly, the prolonged exposure of mouse soleus [236] and primary adult cardiomyocytes [237] to physiological concentrations of βHB causes a marked decrease in insulin stimulated glucose uptake. Notably, this effect only occurred following incubation periods of >9.5 hours, indicating a model of ketone-mediated metabolic adaptation, rather than substrate-level inhibition, as described by the Randle Cycle [14]. This suggests that βHB signalling, independent of the metabolic milieu associated with an endogenous ketosis, might contribute to the metabolic adaptations underlying the insulin resistance and decreased CHO utilisation observed in response to a ketogenic diet or starvation. If true, it is plausible that reciprocal increases in fat oxidation may occur; a finding that would be coherent with the observed increases in intramuscular triglyceride catabolism during an acute exogenously-induced ketosis [71]. This raises the intriguing possibility that supplementing athletes with ketones may, as with a ketogenic diet, amplify the adaptive signal to endurance exercise training.

Therefore, this study sought to compare metabolic adaptations made in response to exercise training during exogenously- and endogenously-induced ketoses in well-trained athletes. It was hypothesised that in common, these distinct physiological states would result in metabolic adaptations that reduce insulin-stimulated glucose disposal and "retool" muscle towards increased fat oxidation. However, in the endogenous ketosis condition only, exercise capacity would be impaired and athletes would demonstrate signs of non-functional overreaching.

5.2 Methods

To address the study goals, athletes were recruited to a partially randomised and blinded, 12-day training and dietary intervention. The three dietary conditions investigated were: (i) exogenous ketosis; (ii) a CHO control condition; and (iii) endogenous ketosis (see study protocol in Figure 5.1).

5.2.1 Subjects and recruitment

Participants were recruited via advertisement and direct invitations to participate. The study protocol was carefully explained to all potential participants, either by phone or in person. Given the challenging nature of adopting a ketogenic diet during exhaustive exercise training, participants were required to actively volunteer for the endogenous ketosis condition (that is, it was non-randomised and non-blinded). Twenty-five participants subsequently enrolled into the study, of which seven volunteered to undertake a ketogenic diet. The remaining 18 were randomly assigned to the exogenous ketosis and CHO conditions following enrolment using a randomisation matrix. Participants in these conditions were blinded to their allocations. Inclusion and exclusion criteria matched those presented in paragraph 2.2. Subjects were actively participating in either cycling, running, triathlon or rowing. All participants had been following a "normal", CHO rich diet for at least two months prior to enrollment (see composition of habitual diets in Table 5.2). Twenty-one participants (n=7 per group) completed the study and were included in data analyses. Participant characteristics, together with group allocation for the four individuals who did not complete the trial, and the reasons for their withdrawal, are presented in Table 5.1. The exogenous ketosis condition included a female athlete, whereas the remaining groups contained males only. Otherwise, no between group differences were observed for participant characteristics.

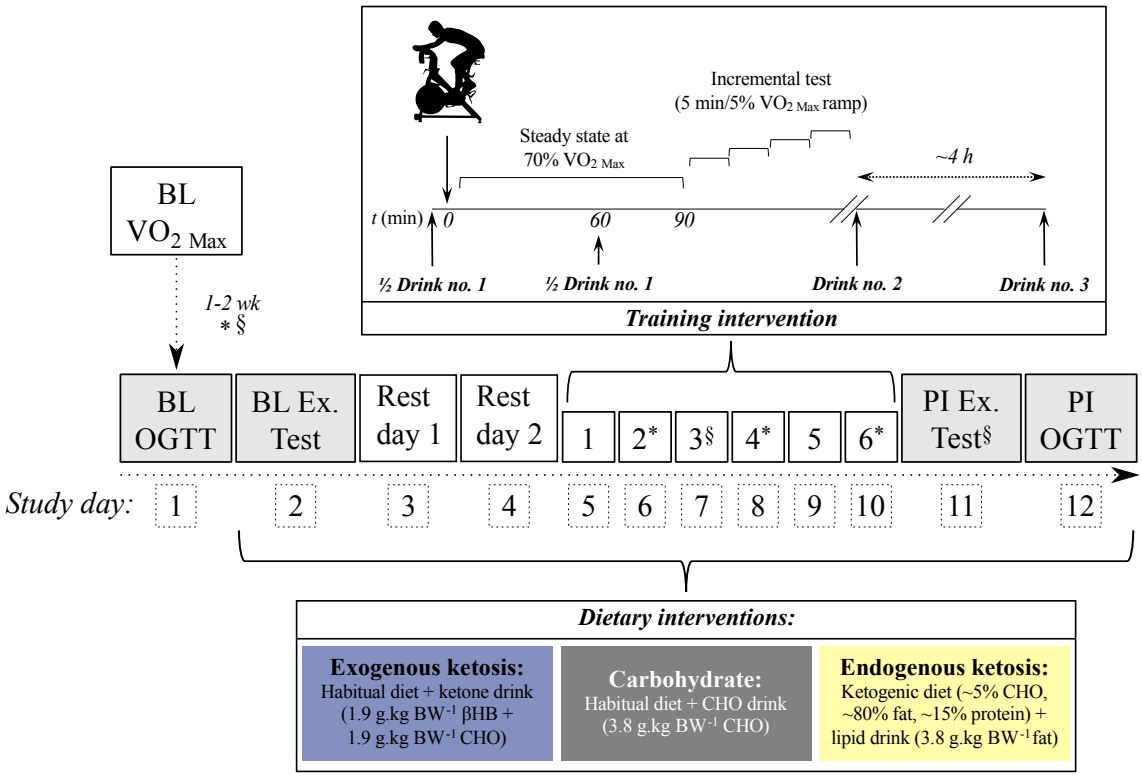


Figure 5.1: **Study protocol overview.** Participants completed metabolic testing visits (highlighted in gray and described in the following paragraphs) before and after an exercise training and dietary intervention. The three experimental diets were: (i) a habitual diet supplemented with a ketone and CHO containing drink (exogenous ketosis, n=7); (ii) a habitual diet supplemented with an isocaloric, CHO only containing drink (CHO, n=7); and (iii) a ketogenic diet supplemented with an isocaloric, fat containing drink (endogenous ketosis; n=7). Dietary interventions began following the baseline exercise (BL Ex.) test and ended after the post intervention (PI) oral glucose tolerance test (OGTT). Participants had two rest days, before commencing a six day structured and supervised training intervention. Participants consumed three study drinks daily. Timings for drink consumption during the training intervention are presented in the Figure. On rest days, drink consumption was aligned approximately with meal-timings (breakfast, lunch and dinner) with a minimum 4 hour gap between each. * = completion of daily analysis of life demands of athletes (DALDA) questionnaire to monitor psychological and physical well-being; § = 24-hour urine collection. Abbreviations not previously defined: BL = baseline; DH = dietary habituation; PI = post intervention.

5.2.2 Baseline exercise test

Following enrollment, subjects completed a baseline $VO_{2\ Max}$ test on an electronically braked cycling ergometer (see paragraph 2.4.1). Power attained at $VO_{2\ peak}$ was recorded and used to set fixed power outputs for the exercise tests and exercise training intervention, such that all participants were performing at a similar relative

Table 5.1: **Participant characteristics.** *p* values represent main condition effects for each characteristic. * = withdrawal due to vasovagal syncope in response to biopsy procedures; § = withdrawal due to illness/injury meaning they could no longer exercise to full exertion. Abbreviations not previously defined: Wt = weight.

	Ex. Ket. (n=7)	**CHO** (n=7)	**End. Ket.** (n=7)	***p***
Gender (m/f)	6/1	7/0	7/0	NA
Age (yr)	25 ± 2	27 ± 7	28 ± 7	0.653
Height (m)	1.89 ± 0.06	1.86 ± 0.06	1.83 ± 0.13	0.415
Weight (kg)	77.6 ± 8.2	82.2 ± 5.2	79.4 ± 12.4	0.672
Body fat (%)	15.8 ± 3.6	17.8 ± 1.8	16.8 ± 4.2	0.578
VO_2 Peak ($L \cdot min^{-1}$)	4.3 ± 1.1	4.5 ± 1.1	4.2 ± 0.7	0.696
Wt adjusted VO_2 Peak ($mL \cdot kg\ BW^{-1}$)	54.7 ± 11.0	58.0 ± 7.3	53.6 ± 8.3	0.676
Power at VO_2 Peak (W)	387 ± 77	367 ± 44	352 ± 49	0.554
Withdrawals	1^{*}	$3^{*,\S x2}$	0	NA

intensity (% of $VO_{2\ peak}$). Participants were given time to adjust the bike according to personal preference before the test. Saddle and handlebar positions were noted and kept constant for the remainder of the study.

5.2.3 Overview of study design

Participants made ten further visits to DPAG over 12 days (Figure 5.1). These consisted of metabolic test days performed before and after the dietary and training intervention. The study protocol sought to blend rigorous scientific control with pragmatic "real-world" allowances necessary for studies requiring high-levels of participant engagement.

5.2.4 Dietary interventions

Dietary interventions commenced immediately after the baseline exercise test and lasted for nine full days thereafter (ending immediately following the post-intervention OGTT; Figure 5.1). The three dietary interventions are summarised below.

1. *Exogenous ketosis*: Participants were asked to maintain a habitual macronutrient dietary composition. They were not given targets for total energy intake.

Participants were supplemented with a ketone monoester and CHO containing drink before, during and after exercise (see the training intervention box in Figure 5.1 for a detailed explanation of timings). The timing of drink delivery sought to achieve an ≈10 - 12 hour period in which blood βHB levels were elevated each day [92]. The drink composition was 393 mg·kg BW^{-1} (target peak blood βHB concentration of ≈4 mM) with an isocaloric amount of dextrose diluted in 400 mL of water. An artificial sweetener (Neotame NutraSweet, Manus Bio, USA) was included to aid palatability. The total energy delivered per drink was 3.8 kcal·kg BW^{-1} (146.4 ± 15.5 kcal per drink of both βHB and dextrose, total kcal = 292.8 ± 30.9).

2. *CHO (control)*: Current nutritional guidelines advocate a high CHO diet to support vigorous exercise training [238]. Accordingly, a CHO control condition was included within this study. As with the exogenous ketosis condition, participants were asked to maintain a habitual macronutrient dietary composition. Study drinks consisted of a weight adjusted isocaloric (to the ketone monoester drink) amount of dextrose diluted in 400 mL water (3.8 kcal·kg BW^{-1}; total kcal = 304.1 ± 19.2) and was delivered at identical times. Drinks contained the same artificial sweetener (Neotame NutraSweet, Manus Bio, USA) and a bitter additive (product code: 648352, Symrise, Germany) to facilitate blinding to study allocations.

3. *Endogenous ketosis:* The target dietary macronutrient composition was ≈5% energy from CHO, ≈80% energy from fat and ≈15% energy from protein, which was expected to induce hepatic ketogenesis [43, 99, 100]. Participants were given a lipid containing drink (Calogen, Nutrica HCP, UK) with a weight adjusted amount of energy to the exogenous ketosis condition drink (3.8 kcal·kg BW^{-1}; total kcal = 293.8 ± 45.9) and delivered at identical times. Participants were provided meals and snacks in order to help them achieve these challenging macronutrient targets. An example daily diet diary from a participant in this condition is provided in Appendix I.

Work sought to investigate the effects of these dietary interventions independent of substantial weight loss (defined as ± 2% change), although a rapid loss of water mass was anticipated following adoption of the ketogenic diet (glycogen being bound to water in an ≈1:3 ratio [239]). Compliance with dietary conditions was determined through self-reported diet diaries. Participants were asked to complete these:

- on two days between enrollment and the baseline OGTT, which was used to represent their habitual diet;
- on one of the two rest days (study days 4 and 5);
- and on two days during the training intervention period (study days 5 to 10).

Participants were educated on how to accurately complete the diaries at the enrolment visit and were given a calibrated set of kitchen scales to measure food mass. Dietary compliance was additionally assessed in the endogenous ketosis condition through the measurement of pre-breakfast capillary blood βHB concentrations, using a portable monitor (Freestyle Optium Neo, Abbott Laboratories, USA) provided to participants at the baseline exercise test. To ensure participants were blinded to condition allocations, βHB concentrations in 24-hour urine collections were used to assess dietary compliance in the exogenous ketosis and CHO conditions.

5.2.5 Training intervention

Participants undertook six days of structured exercise designed to provide a robust training stimulus (that is, to be of sufficient intensity to promote exercise adaptation) and monitor daily exercise capacity. All exercise sessions were undertaken in a controlled laboratory setting at DPAG and were supervised by the same study investigator. Participants were instructed to perform no additional exercise during this time. In nearly all cases, participants attended the laboratory at between 0800 and 1000 to undertake the supervised exercise training. However, occasional allowances were made when this was not feasible due to pre-existing commitments.

Participants were permitted to consume food immediately prior to attending the laboratory. Thus, exercise during the training intervention period was undertaken in the postprandial state. However, no additional calories were permitted upon arrival, other than those provided in study drinks. Participants consumed 50% of a study drink immediately before exercise, with the remainder being consumed 60 min into steady state exercise.

Exercise consisted of 90 min at 70% $VO_{2\ peak}$ on an electronically braked bicycle ergometer (Ergoselect 100, Ergoline, Germany). Following a short comfort break (maximum 10 min), participants then undertook an incremental exercise test. This began at 75% $VO_{2\ peak}$ and increased 5% every 5 min until volitional fatigue or inability to hold a cadence $\geq$60 RPM. Exercise capacity was measured each day and determined as time to exhaustion. Air flow and room temperature were adjusted to each participant's preference and water was allowed ad libitum. Participants were permitted to play music or watch videos throughout exercise and verbal encouragement was given by the study investigator.

Within 5 min of completing exercise, participants consumed a second study drink. Thereafter, participants were permitted any food/drink in accordance with their dietary regime. Before leaving the laboratory, participants were given their final daily study drink, which they were instructed to consume $\approx$4 hours post completion of exercise. Therefore, during the exercise intervention period, a study investigator observed participants ingesting two thirds of the study drinks.

5.2.6 Metabolic testing

In accordance with the study aims, testing sought to determine the effects of dietary and training interventions on resting glucose and exercise metabolism. For all tests, participants attended DPAG having fasted overnight ($\approx$8 - 10 hour fast).

Oral glucose tolerance test

Resting glucose metabolism was measured through an OGTT. Participants attended the laboratory at ≈0800. A catheter was inserted into an antecubital vein of the arm (see paragraph 2.4.3 for a description of the procedure) and a baseline blood sample (≈3 mL) was taken. Participants then consumed a drink containing 75 g dextrose diluted in 400 mL water. Further blood samples were taken at t = 30 min, 60 min, 90 min and 120 min while the participant reclined on a couch. Blood samples were kept in an ice-water bath until the end of the visit.

In a random subsample of participants in the exogenous ketosis (n=3) and CHO (n=3) groups, 500 mg of a glucose isotope tracer (D-Glucose, [U$^{13}C_6$], 99%; Cambridge Isotope Laboratories, USA) was included within the drink, providing a relative enrichment of ≈0.67%. For these participants, respiratory gases were measured by indirect calorimetry (see paragraph 2.4.2) and sampled using a Douglas bag (see paragraph 2.4.2) immediately prior to blood sampling. Collected respiratory gas samples were kept at room temperature until analysis for ^{13}C enrichment by gas chromatography-mass spectrometry (see below). This complementary analysis provides an integrated measurement of glucose metabolism, incorporating glucose absorption, insulin-mediated glucose disposal and cellular glucose oxidation, observed by the presence of $^{13}CO_2$ in breath samples [240].

Exercise tests

Exercise metabolism and physiology was measured during controlled exercise tests (Figure 5.2). Participants attended the laboratory at ≈0800. Body weight and composition were measured using calibrated four-point bioimpedance scales (BF508, Omron, Japan). A catheter was then inserted into an antecubital vein of the arm (see paragraph 2.4.3) and a baseline blood sample (≈3 mL) was drawn. Blood samples were kept in an ice-water bath until the end of the visit. Resting respiratory gases (via indirect calorimetry) and heart rate were recorded (see paragraph 2.4.2). Muscle and subcutaneous adipose tissue biopsies were performed, as detailed in para-

graph 2.4.4. Participants then commenced exercise. Importantly, no study drinks were administered before or during exercise (that is, exercise was performed in the overnight fasted state). As such, any observed differences in exercise metabolism (baseline versus post-intervention exercise tests) may be attributed to the dietary and training intervention. The exercise protocol was identical to that employed for the training intervention (90 min steady state at 70% $VO_{2\ peak}$ followed by an incremental exercise test to volitional fatigue). Blood was drawn (≈3 mL), respiratory gases and heart rate were measured, and perceived exertion was recorded at t = 30 min, 60 min and 90 min during steady state exercise and upon failure during the incremental exercise test. Exercise capacity was determined as time to exhaustion. Air flow and room temperature were adjusted to each participant's preference and water was allowed ad libitum. Verbal encouragement was given by the study investigator during exercise.

Immediately following cessation of exercise, a second muscle biopsy was performed using an incision site ≈3 - 4 cm distal from the first. The approximate time from cessation of exercise to preservation of muscle biopsy samples in liquid nitrogen was 1 - 3 min. Ice was applied intermittently to the muscle biopsy site for ≈20 min whilst the participant recovered. A sterile, waterproof dressing was applied and advice was given to take prophylactic pain killers and/or non-steroidal anti-inflammatory drugs. Participants were instructed not to perform exercise on the two days following the baseline exercise test ("rest days", see Figure 5.1). This rest period was included to give participants an opportunity to recover from the biopsy procedures, and habituate to dietary interventions, before commencing exercise training. Participants consumed study drinks on rest days. Instructions were given to consume one drink with breakfast, lunch and dinner with a minimum 4-hour gap between each.

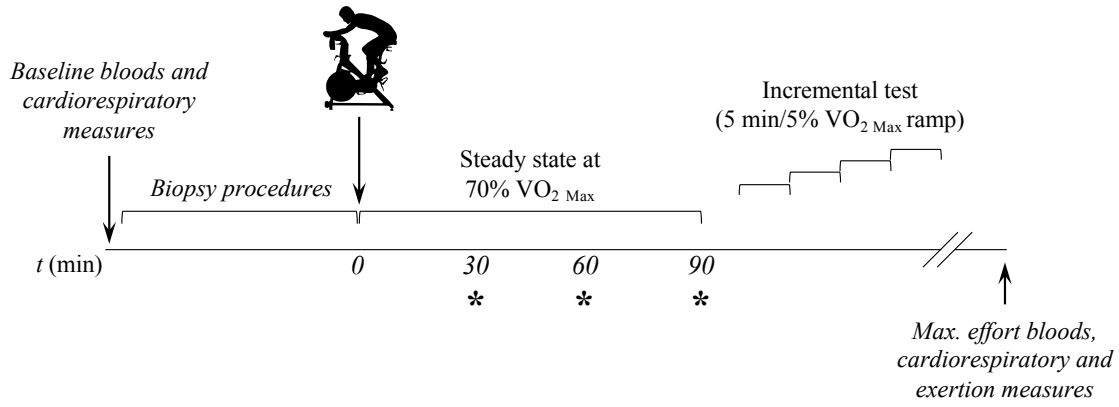


Figure 5.2: **Exercise test protocol.** Participants attended the laboratory following an overnight fast. Muscle and subcutaneous adipose tissue biopsies were performed at rest. A second muscle biopsy was performed immediately following exercise. The exercise protocol was identical to that performed during the training intervention period (90 min at 70% $VO_{2\ peak}$ followed by an incremental exercise test to volitional fatigue (5 min/5% $VO_{2\ peak}$ ramp)). Bloods were taken and cardiorespiratory and exertion measures made at baseline, 30 min, 60 min, 90 min and upon failure during the incremental exercise test. * = blood sampling, cardiorespiratory and exertion measures.

5.2.7 Blood substrate and insulin analyses

βHB was analysed immediately from whole blood samples using a portable analyser (Freestyle Optium Neo, Abbott Laboratories, USA). Following completion of the visit, samples kept on ice were centrifuged for 10 min at 3600 RPM. The plasma component was aliquoted into Eppendorf tubes and these were stored at -80 °C until later analysis. Frozen samples were thawed at room temperature for ≈30 min before biochemical analyses were undertaken. Serum glucose, NEFA and lactate were determined using a calibrated, semi-automated benchtop analyser (Pentra C400, Horiba Medical, France). Serum insulin was assayed using an ELISA kit (product code: 10-1113-01, Mercodia, Sweden). All analyses were performed in duplicate, with the average value taken.

5.2.8 Urine collection and analysis

Participants were asked to collect urine in provided canisters for 24 hours prior to commencing the study ("habitual", performed between enrollment and the baseline

OGTT), on training intervention day 3 and at the post-intervention exercise test day (see Figure 5.1). Two samples (1 mL) were taken and stored at -25 °C until further analysis. To determine adherence with dietary interventions, samples were subsequently thawed at room temperature and analysed for βHB concentration using a calibrated, semi-automated benchtop analyser (Pentra C400, Horiba Medical, France).

5.2.9 Calculation of whole-body substrate oxidation data and gross efficiency

CHO and fat oxidation rates ($g \cdot min^{-1}$) and their contribution to overall energy expenditure (%) were calculated from VCO_2 and VO_2 values using non-protein RER equations [241]. To allow direct comparisons to other studies investigating the effects of a ketogenic diet on exercise substrate metabolism [100–103], calculations were not corrected to account for the effects of ketogenesis and ketolysis [20]. Thus, calculations of substrate oxidation rates include a small systematic error [20].

Energy expenditure was calculated according to the exercise intensity dependent equations proposed by Jeukendrup and Wallis [217].

$$\textit{Energy expenditure at 70\% } VO_{2\ peak} = (0.550 \cdot VCO_2) + (4.471 \cdot VO_2) \quad (5.1)$$

Gross efficiency during steady state exercise was calculated as [73]:

$$\textit{(Work rate (W))/Energy expended } (J \cdot s^{-1}) \textit{.100\%} \quad (5.2)$$

5.2.10 Determination of breath enrichment

The TTR in respired gas samples was used to determine intervention effects on glucose oxidation during the OGTTs and calculated as follows:

$$\textit{Breath enrichment} = TTR\ CO_{2\ exp} - TTR\ CO_{2\ bkg} \quad (5.3)$$

where $TTR_{background}$ represents the enrichment in baseline (pre-drink) samples. Expired CO_2 enrichment was established through gas chromatography-combustion-isotope ratio mass spectrometry, using previously published methods [216].

5.2.11 Training status

The DALDA questionnaire was used to monitor symptoms of non-functional over-reaching. This questionnaire is well validated and has been demonstrated to outperform biochemical markers [242]. Participants completed the DALDA questionnaire prior to commencing the study ("habitual", performed between enrollment and the baseline OGTT) and prior to exercise on training intervention days 2, 4 and 6 (see Figure 5.1).

5.2.12 Intramuscular metabolites

To gain direct insight into the contribution of fat and CHO to energy production at exercise test visits, intramuscular metabolites were measured in pre- and post-exercise muscle samples taken at the baseline and post intervention exercise tests. Detailed methods are provided in paragraph 2.5.2.

5.2.13 Protein-fold changes

To determine any effects of dietary and training interventions on skeletal muscle remodeling, non-targeted proteomics was performed on pre-exercise muscle biopsy samples taken at the baseline- and post-intervention exercise tests. Detailed methods are provided in paragraph 2.5.3.

5.2.14 Intramuscular triglyceride content

Intramuscular triglyceride content was determined in pre-exercise muscle biopsy samples at the baseline and post intervention exercise tests using an ORO staining procedure described by Mehlem and colleagues [163] (see paragraph 2.5.4 for full

methods).

5.2.15 Adipose tissue gene expression

Ketones inhibit adipocyte lipolysis through antagonism of HCAR2, leading to a decrease in cyclic adenosine monophosphate (cAMP) signalling [48, 93]. Prolonged inhibition of HCAR2 through the infusion of nicotinic acid has been demonstrated to increase the expression of genes within the cAMP pathway, leading to a "rebound" in blood FFA concentration and the development of insulin resistance [243]. As such, work sought to determine whether the prolonged inhibition of HCAR2 through elevation of blood βHB concentrations would have a similar effect on gene expression within the cAMP signalling pathway. Accordingly, transcript expression of target genes was measured using qPCR in subcutaneous adipose tissue samples taken at the baseline and post intervention exercise tests (see paragraph 2.5.5 for a full description of methods). Target genes were: diacylglycerol O-acyltransferase 2 (DGAT2) (primer ID = Hs01045913 m1, Life Technologies, UK), hormone sensitive lipase (HSL) (Hs00943410 m1), phosphodiesterase 3B (PDE3B) (Hs00610035 m1), perilipin 1 (PLIN1) (Hs00160173 m1) and solute carrier family 2, facilitated glucose transporter member 4 (GLUT 4) (Hs01586776 m1).

5.2.16 Statistics

All data reported are mean ± SD and statistical significance was taken as $p<0.05$. The statistical tests employed are reported in the Results section.

5.3 Results

5.3.1 Dietary compliance

In comparison to their habitual diet, subjects in the endogenous ketosis group significantly reduced their CHO consumption during the rest and training intervention

periods (both $p<0.001$), which represented just ≈4% of total energy intake (Table 5.2). Consequently, the proportion of calories from fat increased more than 2-fold to ≈84% during the rest and training intervention periods ($p<0.001$; see Appendix I for an example diet diary).

In the CHO condition, there were equal-in-magnitude but opposite-direction effects on fat and CHO intake during the training intervention period, which were significantly decreased and increased by ≈12%, respectively. In the exogenous ketosis condition, βHB accounted for ≈11% of total energy intake. Despite the addition of this fourth major macronutrient, the proportion of energy derived from CHO, fat and protein was similar versus the participants' habitual diets.

Abstinence from dietary CHO was sufficient to stimulate hepatic ketogenesis in the endogenous ketosis group. Overnight fasted capillary βHB concentrations were significantly greater versus the baseline exercise test from day three onward (all $p<0.001$), peaking at 4.1 ± 1.4 mM at the post-intervention exercise test (Figure 5.3 A). βHB concentrations in 24-hour urine collections were significantly greater in the exogenous and endogenous ketosis conditions versus the CHO condition during the dietary and training intervention (Figure 5.3 B). Notably, urinary βHB concentrations were ≈4-fold higher in the endogenous (peak = 17.6 ± 12.3 mM) versus exogenous ketosis (peak = 4.2 ± 2.3 mM) conditions on day 3 ($p=0.016$) and post-intervention exercise test ($p=0.044$) collections. Capillary βHB concentrations were measured before and during exercise for participants in the exogenous ketosis and CHO groups on training intervention day 1. Coherent with the ketone monoester supplementation timing (that is, 50% of the drink being ingested before exercise and the remainder at t = 60 min, see Figure 5.1) two peaks in blood βHB were observed (Figure 5.3 C). Blood βHB concentration was significantly greater in the exogenous ketosis versus CHO condition at t = 30 and 90 min, and at exhaustion (Max). Taken together, these data indicate that participants adhered to group specific dietary interventions.

Table 5.2: **Dietary composition**. Data taken from self-reported diet diaries. Statistical comparisons were made by 2-way RM ANOVA. Significant post-hoc comparisons (highlighted in gray): * = CHO intake versus habitual; $ = fat intake versus habitual. Acronyms not previously defined: TEI = total energy intake.

	Habitual				**Rest**					**Training intervention**				
	TEI (kcal)	CHO (%)	Fat (%)	Prot. (%)	TEI (kcal)	CHO (%)	Fat (%)	Prot. (%)	βHB (%)	TEI (kcal)	CHO (%)	Fat (%)	Prot. (%)	βHB (%)
Exogenous ketosis	2907 ± 725	52.7 ± 13.9	29.5 ± 10.4	17.8 ± 4.2	3624 ± 985	51.2 ± 4.6	24.9 ± 5.9	12.3 ± 2.0	12.3 ± 2.4	4318 ± 1050	49.5 ± 10.1	27.6 ± 9.3	12.4 ± 2.3	10.3 ± 1.9
CHO	3988 ± 1308	53.5 ± 4.7	34.0 ± 2.2	12.4 ± 4.4	4152 ± 1220	61.5 ± 4.7	24.5 ± 5.0	13.9 ± 0.5	NA	3967 ± 660	65.72 ± 7.69*	21.8 ± 4.7$	12.6 ± 3.0	NA
Endogenous ketosis	3660 ± 719	48.5 ± 9.4	35.7 ± 10.0	15.7 ± 3.5	3701 ± 451	4.5 ± 0.9*	84.0 ± 2.5$	11.5 ± 2.7	NA	4118 ± 945	4.2 ± 1.8*	83.5 ± 4.6$	12.2 ± 3.5	NA

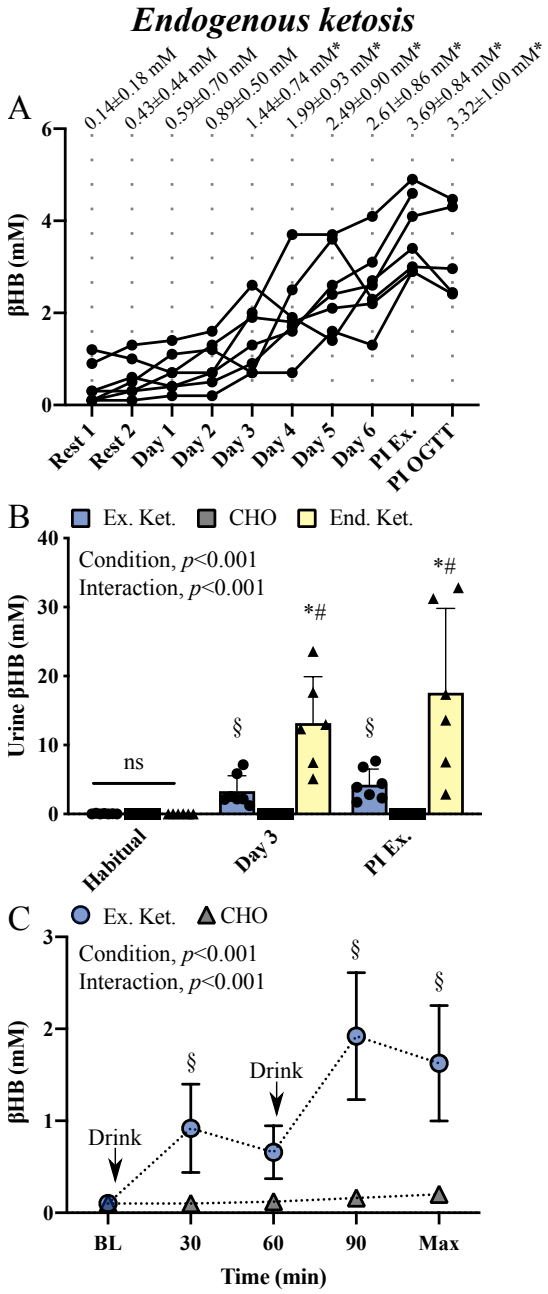


Figure 5.3: **Blood and urine βHB concentration.** **(A)** Overnight fasted capillary blood βHB concentrations in the endogenous ketosis condition. Mean ± SD concentrations are provided above. **(B)** 24 h urine βHB concentrations. **(C)** Capillary blood βHB concentration at rest and during exercise on training intervention day 1. Changes in capillary βHB concentrations were analysed by 1-way ANOVA. * = significant post-hoc comparison versus baseline exercise test (BL Ex.). Statistical comparisons of urine and capillary βHB concentrations were made by 2-way RM ANOVA. Significant post-hoc comparisons: § = exogenous ketosis and CHO groups; * = exogenous versus endogenous ketosis; # = CHO versus endogenous ketosis. Abbreviations not previously defined: PI Ex. = post-intervention exercise test.

5.3.2 Body weight

Participants in the endogenous ketosis group lost significantly more body weight (3.1 ± 1.0 kg) versus their counterparts in the exogenous ketosis and CHO groups (both p<0.001; Figure 5.4 A). Notably, ≈60% of this weight loss occurred on study day 1 (baseline exercise test day; Figure 5.4 B), likely reflecting a loss of water mass due to a depletion (and inadequate post-exercise recovery) of intramuscular glycogen [239].

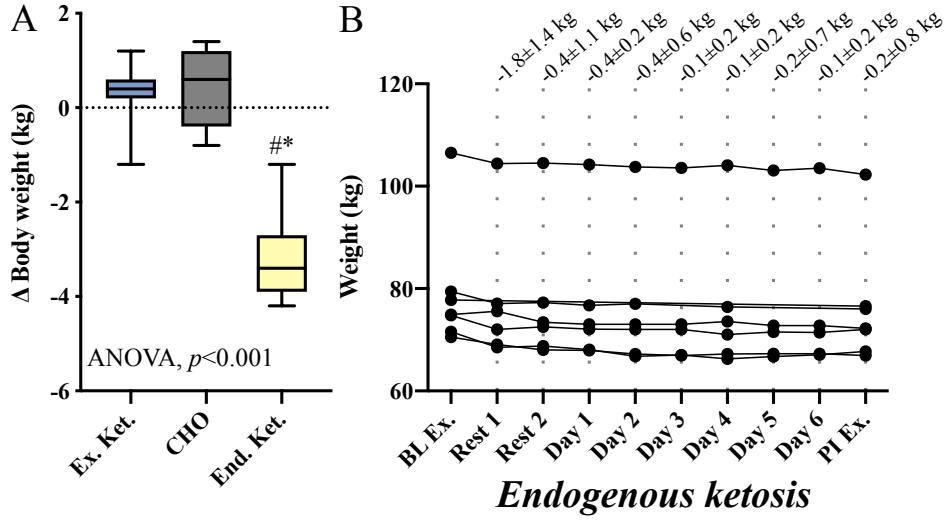


Figure 5.4: **Body weight and fat mass. (A)** The effect of dietary and exercise interventions on participant body weight measured at the baseline and post intervention exercise tests. **(B)** Overnight fasted body weight measurements for subjects in the endogenous ketosis condition. Mean ± SD daily changes in body weight are provided above. Statistical comparisons of delta values (post-intervention - baseline measurements) were made by 1-way ANOVA. Significant post-hoc comparisons: * = endogenous ketosis versus exogenous ketosis; # = endogenous ketosis versus CHO.

5.3.3 Resting glucose metabolism

Fasting blood glucose and insulin

Fasting blood glucose concentrations tended to increase from 5.88 ± 1.17 mM to 6.43 ± 1.21 mM (p=0.050) following the dietary and training intervention in the exogenous ketosis condition (Figure 5.5 A). Delta fasted glucose was significantly greater in the exogenous ketosis versus endogenous ketosis condition (p=0.010) (Figures 5.5

D). Neither fasting insulin (Figures 5.5 E - H) nor homeostatic model assessment of insulin resistance (HOMA IR) (data not shown) were affected by the dietary interventions.

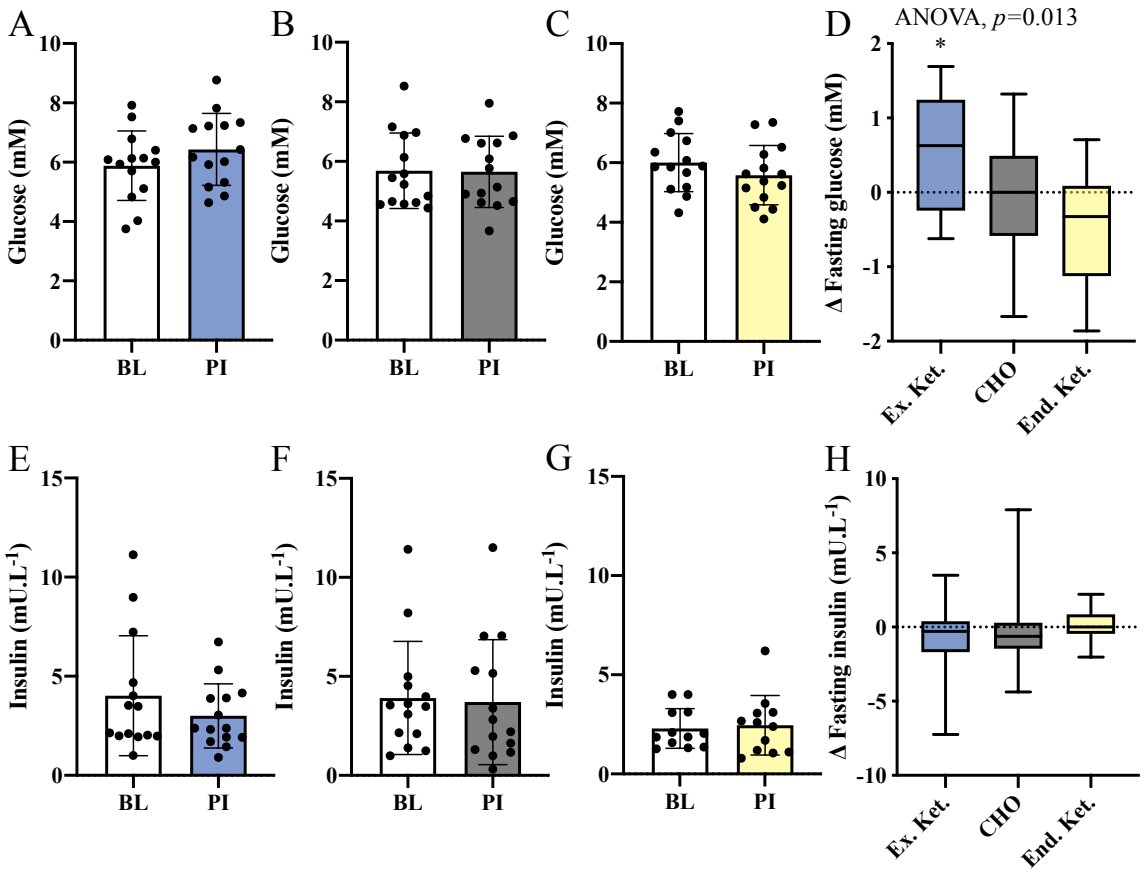


Figure 5.5: **Fasted blood glucose and insulin.** Fasting blood glucose concentrations at baseline (BL) and post-intervention (PI) (including fasting values measured at the OGTT and exercise test) in the **(A)** exogenous ketosis, **(B)** CHO and **(C)** endogenous ketosis conditions. **(D)** Between group comparison of delta fasting blood glucose. Fasting blood glucose concentrations were higher than expected across all groups both pre- and post-intervention. This is likely attributable to a systematic bias (that is, consistent to all glucose measurements) introduced by the apparatus used to measure glucose concentrations. Fasting blood insulin concentrations at baseline and post-intervention (including fasting values measured at the OGTT and exercise test) in the **(E)** exogenous ketosis, **(F)** CHO and **(G)** endogenous ketosis conditions. **(H)** Between group comparison of delta fasting blood insulin. Within group comparisons of fasting blood glucose and insulin were investigated by 2-way RM ANOVA. Between group comparisons of delta glucose (post-intervention - baseline values) were investigated by 1-way ANOVA. Between group comparisons of delta insulin were investigated by Kruskal-Wallis test. Significant post-hoc comparisons: Ψ = post-intervention versus baseline; * = exogenous ketosis versus endogenous ketosis.

Oral glucose tolerance test

Supplementing athletes with βHB markedly reduced insulin sensitivity, observed as a significant increase in blood glucose (p<0.001) and insulin (p=0.003) curves in response to a 75 g oral glucose load (Figures 5.6 A and B). Notably, despite the increase in glucose and insulin concentrations, all measurements remained within the normal physiological range (that is, they did not meet the diagnostic criteria for insulin resistance). As such, a relative rather than absolute insulin resistance was observed. Conversely, insulin sensitivity was not affected by either the CHO or endogenous ketosis interventions (Figures 5.6 C - F). Accordingly, delta glucose AUC was significantly greater in the exogenous ketosis versus CHO (p=0.002) and endogenous ketosis (p=0.016) conditions (Figure 5.6 G). However, delta insulin AUC only tended to be greater versus the CHO (p=0.053) and endogenous ketosis (p=0.094) conditions (Figure 5.6 H).

Given that no study drinks were consumed prior to the OGTT (thus adaptations to the dietary interventions were being tested, rather than the acute effects of ketone monoester ingestion), baseline blood βHB concentration was significantly elevated at the post-intervention OGTT in the endogenous ketosis versus exogenous ketosis and CHO conditions (both p<0.001; Figure 5.7).

Glucose oxidation

A ^{13}C label was included in glucose drinks for a random subset of individuals (n=3 per group) in the exogenous ketosis and CHO conditions. This provided a complementary measure of resting glucose metabolism that incorporated cellular glucose respiration [240]. A visit-time interaction effect was observed for respiratory gas TTRs in the exogenous ketosis condition, with the 120 min measurement being significantly lower at the post-intervention versus baseline OGTT (p=0.006; Figure 5.8 A). Conversely, a main effect of visit was observed in the CHO condition, with respiratory gas TTRs being significantly higher at the post-intervention versus baseline OGTT (Figure 5.8 B). Thus, participants in the CHO group had increased resting

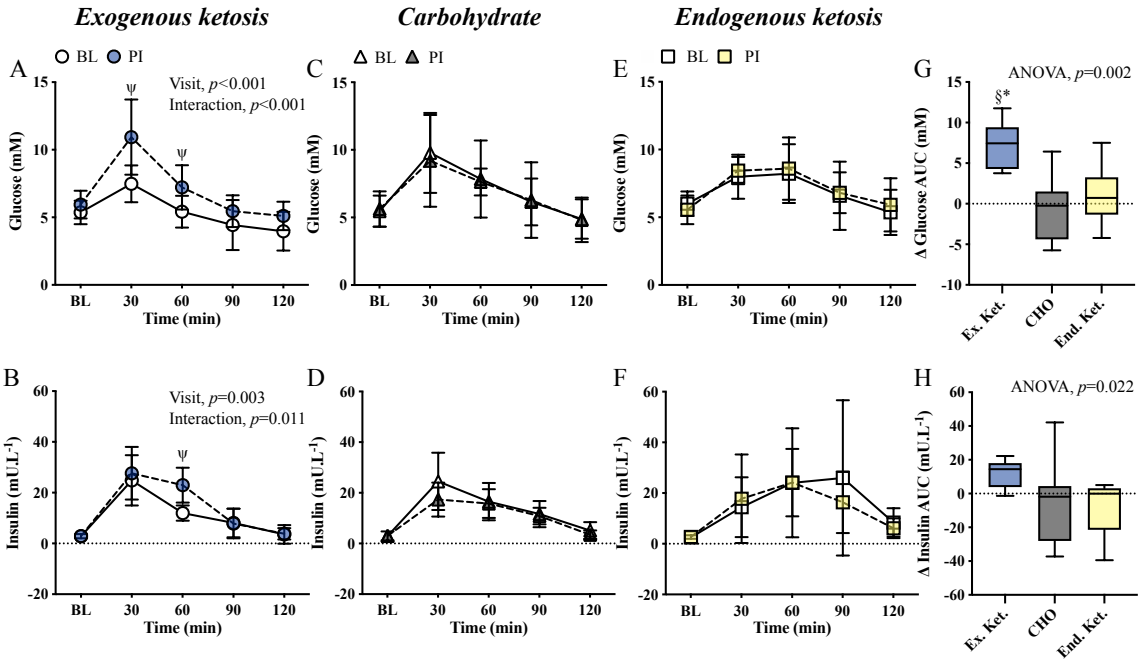


Figure 5.6: **Glucose tolerance test.** Baseline (BL, open shape with solid lines) and post-intervention (PI, closed shapes with dotted lines) blood glucose curves in response to a 75 g glucose containing drink in the **(A)** exogenous ketosis, **(C)** CHO and **(E)** endogenous ketosis groups. **(G)** Between group comparison of delta glucose AUC. Baseline (open shape with solid lines) and post-intervention (closed shapes with dotted lines) blood insulin curves in response to a 75 g glucose containing drink in the **(B)** exogenous ketosis, **(D)** CHO and **(F)** endogenous ketosis groups. **(H)** Between group comparison of delta insulin AUC. Within group comparisons of blood glucose and insulin curves were investigated by 2-way RM ANOVA. Significant post-hoc comparisons: Ψ = post-intervention versus baseline at the indicated measurement. Between group comparisons of delta glucose and insulin AUC (post-intervention - baseline values) were investigated by 1-way ANOVA and Kruskal-Wallis test, respectively. Significant post-hoc comparisons: § = exogenous ketosis versus CHO; * = exogenous ketosis versus endogenous ketosis.

glucose oxidation in response to a 75 g oral glucose load following the dietary and exercise intervention. Accordingly, delta TTR values were significantly greater in the CHO versus exogenous ketosis conditions at 60 min, 90 min and 120 min (all $p<0.001$, Figure 5.8 C). Finally, delta TTR was significantly negatively correlated with delta blood glucose (also measured at the OGTTs), such that changes in glucose oxidation (reflected by a low TTR value) accounted for ≈24% of variance in blood glucose concentrations (Figure 5.8 D).

In sum, supplementing a habitual diet with a ketone monoester and CHO containing drink impaired glucose tolerance. However, no differences were observed

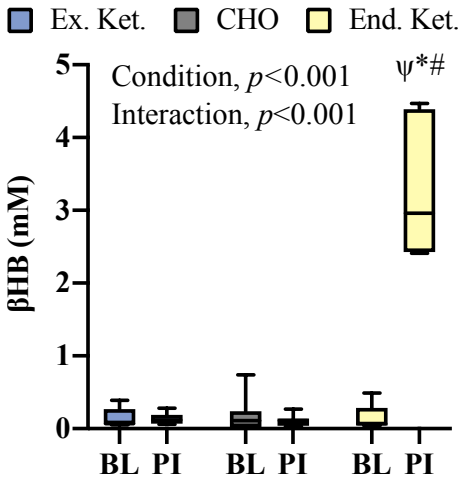


Figure 5.7: **Baseline (fasted) blood βHB concentration at the OGTTs.** Overnight fasted blood βHB concentrations at baseline (BL) and post-intervention (PI) OGTTs. Between group comparisons were investigated by 2-way RM ANOVA. Significant post-hoc comparisons: Ψ = versus baseline measurement (within group); * = versus equivalent visit in the exogenous ketosis condition; # versus equivalent visit in the CHO condition.

in the endogenous ketosis condition. Whilst glucose and insulin measurements remained within the normal physiological range in response to a 75 g oral glucose load (that is, the diagnostic criteria was not met), the observed effects of prolonged ketone monoester ingestion on glucose tolerance are striking given the cohort of individuals studied (physically active, well-trained, healthy individuals) and that it occurred despite undertaking vigorous exercise training, which is insulin sensitising [244].

5.3.4 Exercise capacity

Exercise capacity was assessed in two ways: (i) baseline test versus post-intervention test time to exhaustion (both performed in an overnight fasted state and where athletes *were not* supplemented with study drinks before or during exercise); and (ii) baseline test time to exhaustion versus daily time to exhaustion during the training intervention period (where, in the latter, athletes performed in the postprandial state and were supplemented with study drinks before and during exercise).

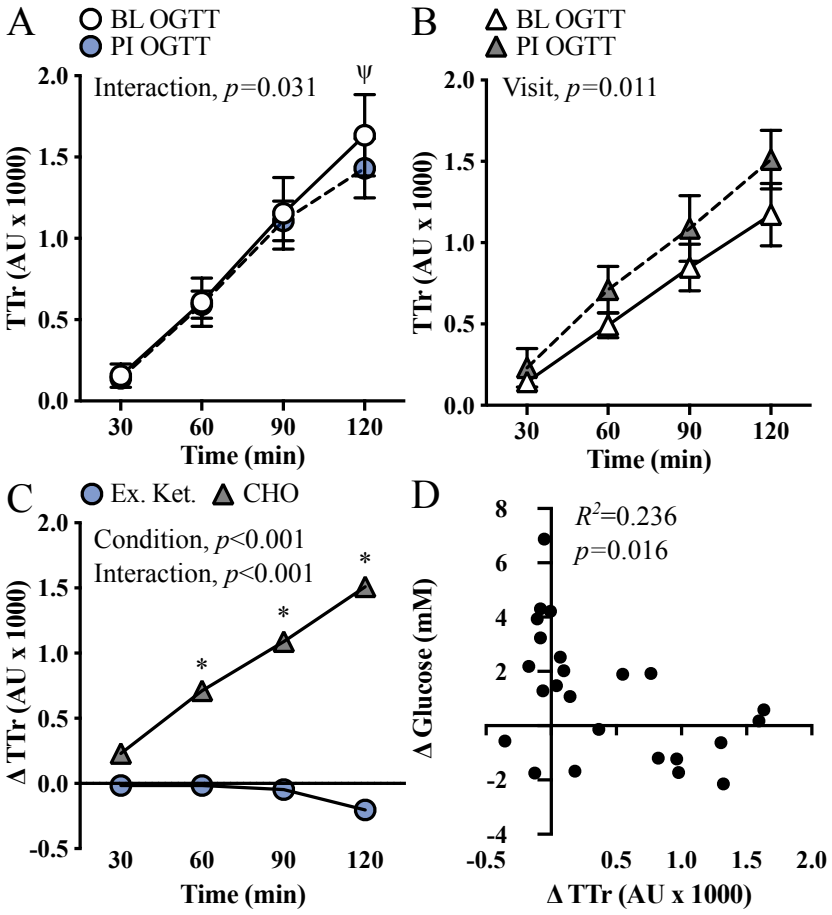


Figure 5.8: **Glucose respiration during the oral glucose tolerance test.** Baseline (BL, open shape with solid lines) and post-intervention (PI, closed shapes with dotted lines) respiratory gas TTRs in response to a 75 g ^{13}C labelled glucose containing drink in the **(A)** exogenous ketosis and **(B)** CHO groups (n=3 per group). **(C)** Delta TTR (post-intervention - baseline values) for the exogenous ketosis and CHO conditions. Positive values represent an increase in glucose oxidation during the post-intervention OGTT. **(D)** Correlation between delta TTR and delta blood glucose. Within group comparisons of respiratory gas TTRs and between group comparisons of delta TTRs were investigated by 2-way RM ANOVA. Significant post-hoc comparisons: Ψ = post-intervention versus baseline at the indicated measurement; * = CHO and exogenous ketosis at the indicated measurement. The relationship between delta TTR and delta glucose was investigated through simple linear regression.

Post-intervention versus baseline exercise test capacity

Exercise capacity was reduced ≈35% at the post-intervention versus baseline exercise test in the endogenous ketosis condition (p<0.001; Figure 5.9 C). Conversely, no differences were observed in either the exogenous ketosis or CHO conditions (Figures 5.9 A and B). Consequently, delta time to exhaustion was significantly lower in

the endogenous ketosis versus exogenous ketosis (p<0.029) and CHO (p=0.006) conditions (Figure 5.9 D).

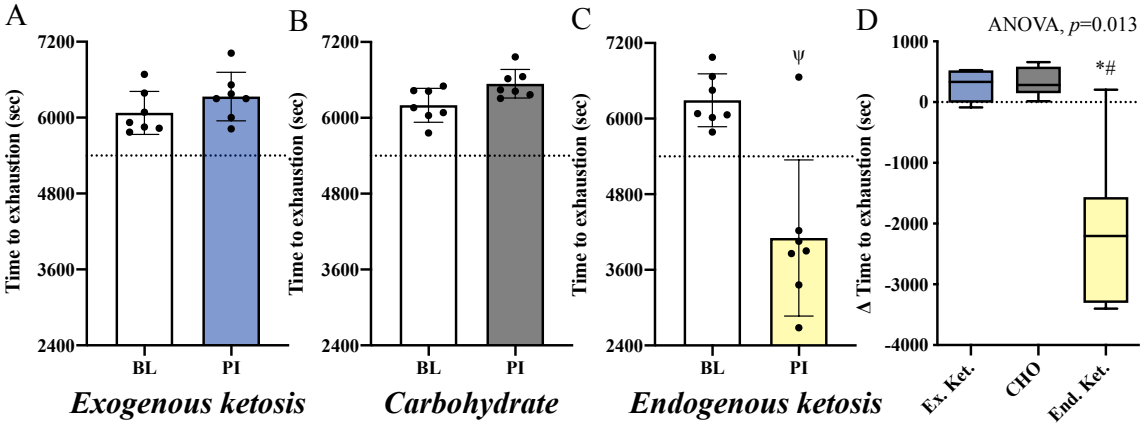


Figure 5.9: **Exercise capacity at the baseline and post-intervention exercise tests.** Exercise capacity at the baseline (BL) and post-intervention (PI) exercise tests for subjects in the **(A)** exogenous ketosis, **(B)** CHO and **(C)** endogenous ketosis groups. **(D)** Between group comparison of delta exercise capacity (post-intervention - baseline time to exhaustion). Within group comparisons of exercise capacity were investigated by 2-way RM ANOVA. Significant post-hoc comparisons: Ψ = post-intervention versus baseline. Between group comparisons of delta exercise capacity were investigated by Kruskal-Wallis test. Significant post-hoc comparisons: * = endogenous versus exogenous ketosis; # = endogenous ketosis versus CHO.

Exercise capacity during the training intervention period

Exercise capacity versus baseline was significantly improved on days 1, 4, 5 and 6 in the exogenous ketosis group (Figure 5.10 A) and significantly worse on all days in the endogenous ketosis group (Figure 5.10 C). Notably, just one subject in the endogenous ketosis group managed to complete the 90 min steady state exercise during the training intervention period, which they achieved on three occasions. No differences in exercise capacity versus baseline were observed in the CHO condition (Figure 5.10 B).

Delta time to exhaustion (daily time to exhaustion - baseline time to exhaustion) was significantly greater in the exogenous ketosis and CHO groups versus the endogenous ketosis group on all training days (Figure 5.10 D). Notably, delta time to exhaustion was also significantly greater in the exogenous ketosis versus CHO condition on days 1, 4 and 5 (Figure 5.10 D).

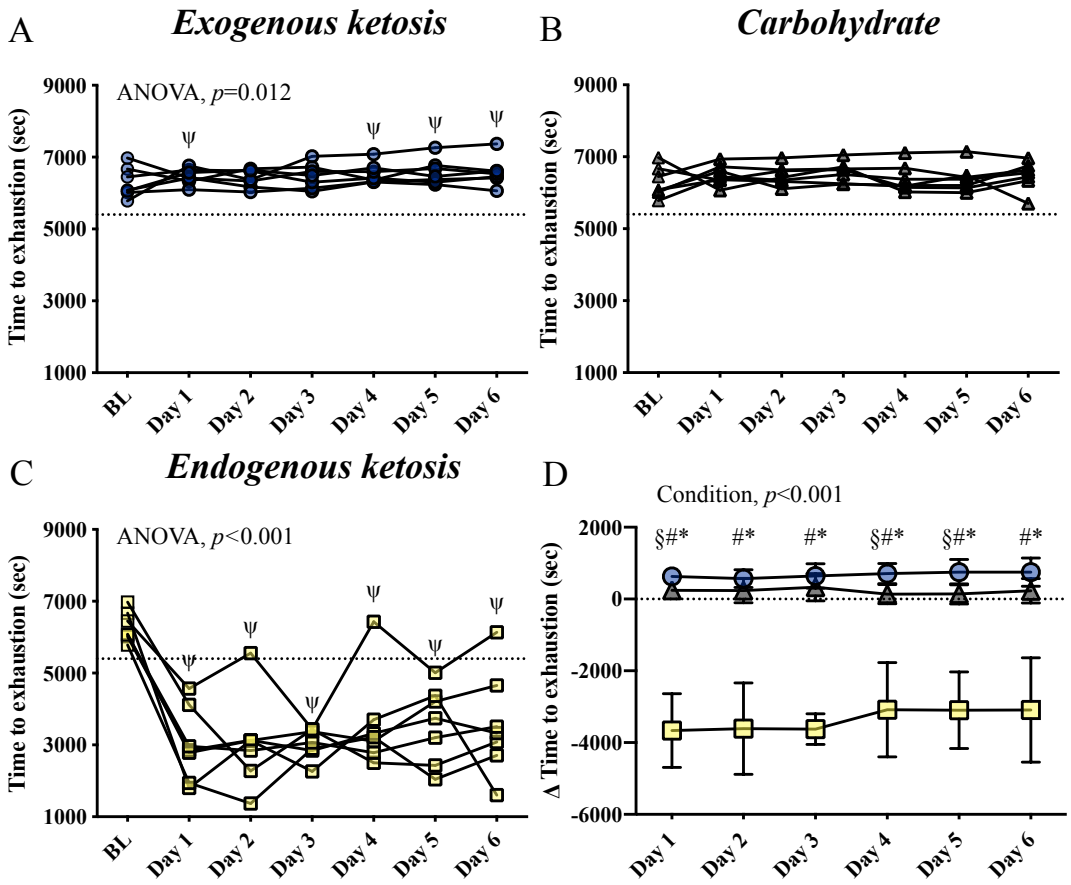


Figure 5.10: **Exercise capacity during the training intervention period.** Exercise capacity during the training intervention period for subjects in the **(A)** exogenous ketosis, **(B)** CHO and **(C)** endogenous ketosis groups. **(D)** Between group comparison of delta exercise capacity (daily time to exhaustion - baseline time to exhaustion). Within group comparisons of exercise capacity were investigated by 1-way ANOVA. Significant post-hoc comparisons: Ψ = the indicated training day versus baseline. Between group comparisons of delta exercise capacity were investigated by 2-way RM ANOVA. Significant post-hoc comparisons: § = exogenous ketosis versus CHO; * = exogenous versus endogenous ketosis; # = CHO versus endogenous ketosis.

In sum, exercise capacity declined substantially following adoption of a ketogenic diet, both in relation to athletes' baseline capacity and in comparison to other dietary conditions. Conversely, modest increases in exercise capacity were observed in the exogenous ketosis group during the training intervention period, again, both versus baseline capacity and versus other conditions. Notably, subjects in the exogenous ketosis group significantly outperformed their counterparts in the CHO group on 50% of training days.

5.3.5 Blood substrate concentrations and the cardiorespiratory response to exercise

Blood substrate and cardiorespiratory data were measured in the overnight fasted state at the baseline and post-intervention exercise tests. Thus, any differences were expected to reflect metabolic adaptation to the dietary and exercise interventions. This data was analysed in two ways.

1. *Steady state exercise*: Delta values (post-intervention - baseline exercise test values) at $t = 0$ min, 30 min and 60 min during steady state exercise were compared between the three dietary conditions using 2-way RM ANOVA or Kruskal-Wallis test. The $t = 90$ min values were ignored due to just one participant reaching this time point at the post-intervention exercise test in the endogenous ketosis condition (see Figure 5.9).

2. *Max effort*: Delta values at max effort (that is, for blood samples taken immediately upon cessation of incremental-intensity exercise) in the exogenous ketosis and CHO groups were analysed separately by paired t-test or Wilcoxon matched-pairs signed rank test.

Full blood substrate and cardiorespiratory data are presented in Appendices J and K.

Blood substrate concentrations

No between group differences were observed for delta blood lactate (Figure 5.11 A). There was a main effect of condition for delta glucose concentration (p=0.010), which was lower in the endogenous ketosis versus exogenous ketosis (p=0.024) and CHO (p=0.016) conditions (Figure 5.11 B). A main effect of condition was present for delta NEFA concentration (p=0.014), which was significantly greater in the endogenous ketosis versus exogenous ketosis (p=0.021) and CHO (p=0.036) conditions (Figure 5.11 C). Finally, there was a main effect of condition (p=0.002) and condition-time

interaction effect (p<0.001) for delta triglyceride concentration, which was elevated in the endogenous ketosis versus exogenous ketosis and CHO conditions at t = 30 min (p=0.010 and p=0.027, respectively) and 60 min (both p<0.001) (Figure 5.11 D). Baseline blood βHB concentration was significantly elevated at the post-intervention exercise test in the endogenous ketosis versus exogenous ketosis and CHO conditions (both p<0.001; Figure 5.12). Given that athletes did not ingest study drinks at the baseline or post-intervention exercise test visits (thus, metabolic adaptations to the dietary and training interventions were being measured, rather than the acute effects of ingesting the study drinks), blood βHB concentrations in the exogenous ketosis and CHO conditions were negligible. Full blood substrate data are presented in Appendix J.

Cardiorespiratory measures, substrate oxidation rates and gross efficiency

Delta VO_2 was similar between all groups (Figure 5.13 A). There tended to be a main condition effect (p=0.071) and condition-time interaction effect (p=0.054) for delta V_E, which was significantly lower at t = 30 min in the CHO versus exogenous ketosis condition (p=0.014) and at t = 60 min in the CHO versus endogenous ketosis condition (p=0.046) (Figure 5.11 B). There was a significant condition effect for delta RER (p=0.010), which was lower in the endogenous ketosis versus CHO condition (p=0.010) and tended to be lower in the endogenous ketosis versus exogenous ketosis condition (p=0.051) (Figure 5.11 C). Predicted fat oxidation rates were increased ≈64% during steady state exercise in the endogenous ketosis condition, from 0.42 ± 0.18 g·min^{-1} at the baseline exercise test to 1.17 ± 0.41 g·min^{-1} at the post-intervention exercise test (Figure 5.14 A). No differences were observed for gross efficiency during steady state exercise (Figure 5.14 B). Finally, there was a significant condition effect (p=0.009) and a condition-time interaction effect (p<0.001) for delta HR, which was greater in the endogenous ketosis versus exogenous ketosis (p=0.019) and CHO conditions (p<0.001) at t = 60 min (Figure 5.11 D). Full cardiorespiratory data are presented in Appendix K.

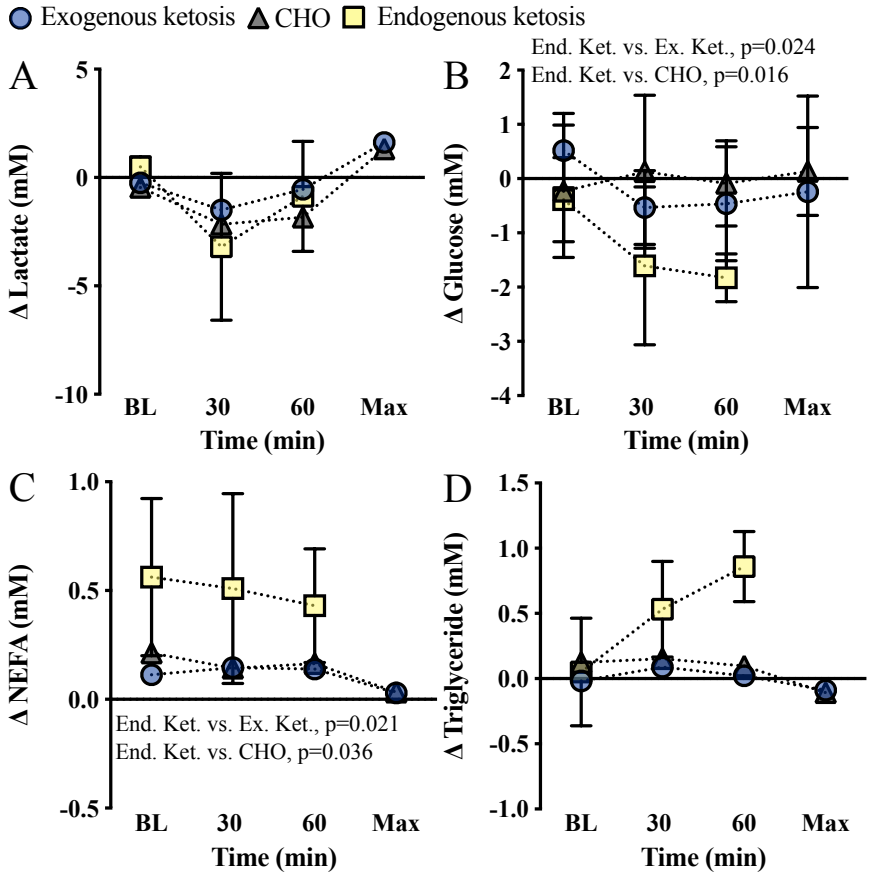


Figure 5.11: **Delta blood substrate concentrations.** Delta blood **(A)** lactate, **(B)** glucose, **(C)** NEFA and **(D)** triglyceride concentrations (post-intervention exercise test values - baseline exercise test values). Baseline (BL), and $t = 30$ min and 60 min values were analysed via 2-way RM ANOVA using data from all three dietary conditions. Max values for exogenous ketosis and CHO conditions were analysed separately by Student's t-test. Significant post-hoc comparisons: * = exogenous versus endogenous ketosis; # = CHO versus endogenous ketosis.

5.3.6 Intramuscular metabolites

Intramuscular metabolites were measured from vastus lateralis biopsy samples taken pre-exercise and upon volitional fatigue at the baseline and post-intervention exercise tests. Metabolite fold-changes were determined as:

(post-exercise abundance - pre-exercise abundance)/pre-exercise abundance.

These were subsequently analysed by by 2-way RM ANOVA, with the following post-hoc comparisons applied:

1. within-group comparisons of metabolite fold-changes (simple effects of visit);

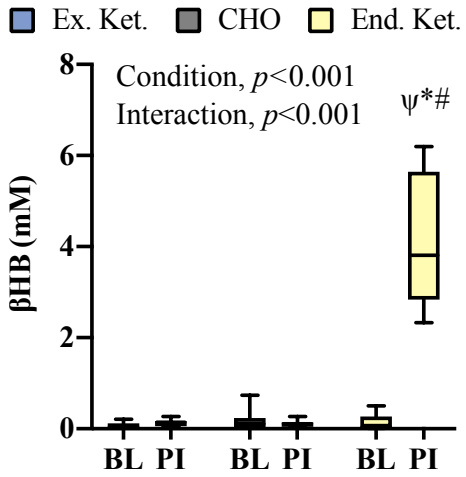


Figure 5.12: **Baseline (fasted) blood βHB concentration at exercise tests.** Overnight fasted blood βHB concentrations at baseline (BL) and post-intervention (PI) exercise tests. Between group comparisons were investigated by 2-way RM ANOVA. Significant post-hoc comparisons: Ψ = versus baseline measurement (within group); * = versus equivalent visit in the exogenous ketosis condition; # versus equivalent visit in the CHO condition.

2. and between-group comparisons at each visit (simple effect of condition).

Full intramuscular metabolite profiles are presented in Appendix L.

Intramuscular glycolytic and tricarboxylic acid cycle intermediaries

The fold-change for the sum of glycolytic intermediates was significantly lower at the post-intervention exercise test between endogenous ketosis and CHO conditions (p=0.032; Figure 5.15 A). This effect was driven by changes in fructose 6-phosphate (F6P) abundance, which was the only individual glycolytic intermediate to be altered (p=0.014; Figure 5.15 B). No other within-group or between-group differences were observed for fold changes in glycolytic intermediates (Figures 5.15 D - F).

In the CHO condition, the fold-change in 2-oxoglutaric acid at the post-intervention exercise test was significantly greater versus the baseline exercise test (p=0.017), and versus the equivalent measure in the exogenous ketosis condition (p=0.046) (Figure 5.15 H). No other differences were observed for fold changes in TCA cycle metabolites (Figures 5.15 F - G, I - J).

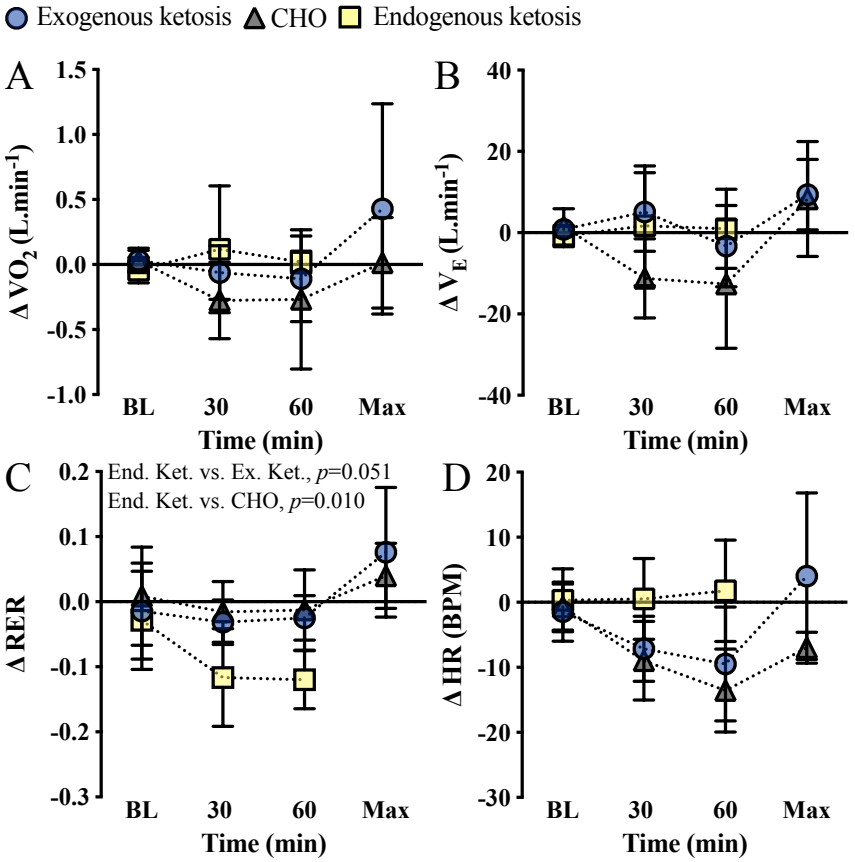


Figure 5.13: **Delta cardiorespiratory measures.** **(A)** Delta VO_2 (post-intervention exercise test values - baseline exercise test values). **(B)** Delta V_E. **(C)** Delta RER. **(D)** Delta HR. Baseline (BL), and $t = 30$ min and 60 min values were analysed via 2-way RM ANOVA using data from all three dietary conditions. Max values for exogenous ketosis and CHO conditions were analysed separately by Student's t-test. Significant post-hoc comparisons: § = exogenous ketosis versus CHO; * = exogenous ketosis versus endogenous ketosis; # = CHO versus endogenous ketosis.

Intramuscular carnitine derivatives

No within-group or between-group differences were observed for fold changes in carnitine derivatives (Figures 5.16 A - F).

5.3.7 Intramuscular lipid content

No differences were observed for intramuscular triglyceride content in muscle biopsy samples taken pre-exercise at the baseline and post-intervention exercise tests (Figure 5.17).

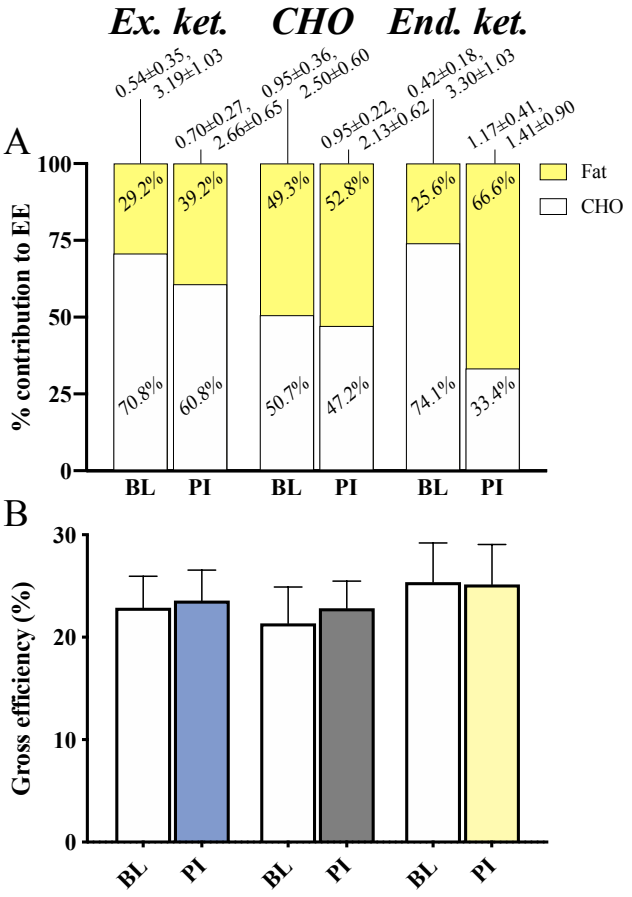


Figure 5.14: **Substrate oxidation and gross efficiency during steady-state exercise. (A)** Percentage contribution of fat and CHO respiration to overall energy expenditure (EE) at the baseline (BL) and post-intervention (PI) exercise tests. Mean ± SD fat and CHO oxidation rates ($g \cdot min^{-1}$) predicted using non-protein equations [241] are indicated above. **(B)** Gross efficiency during steady state exercise at the baseline and post-intervention exercise tests. Gross efficiency was analysed via 2-way RM ANOVA. Significant post-hoc comparisons: # = CHO versus endogenous ketosis at the indicated visit.

5.3.8 Mood state and exercise exertion

Daily analysis of life demands of athletes

There was a significant main effect of condition for athletes' responses to the DALDA questionnaire (p=0.022). Athletes in the endogenous ketosis group recorded more "worse than normal" responses (representing increased mood disturbance) during the training intervention period versus participants in the exogenous ketosis (p=0.046) and CHO (p=0.028) conditions (Figure 5.19 A).

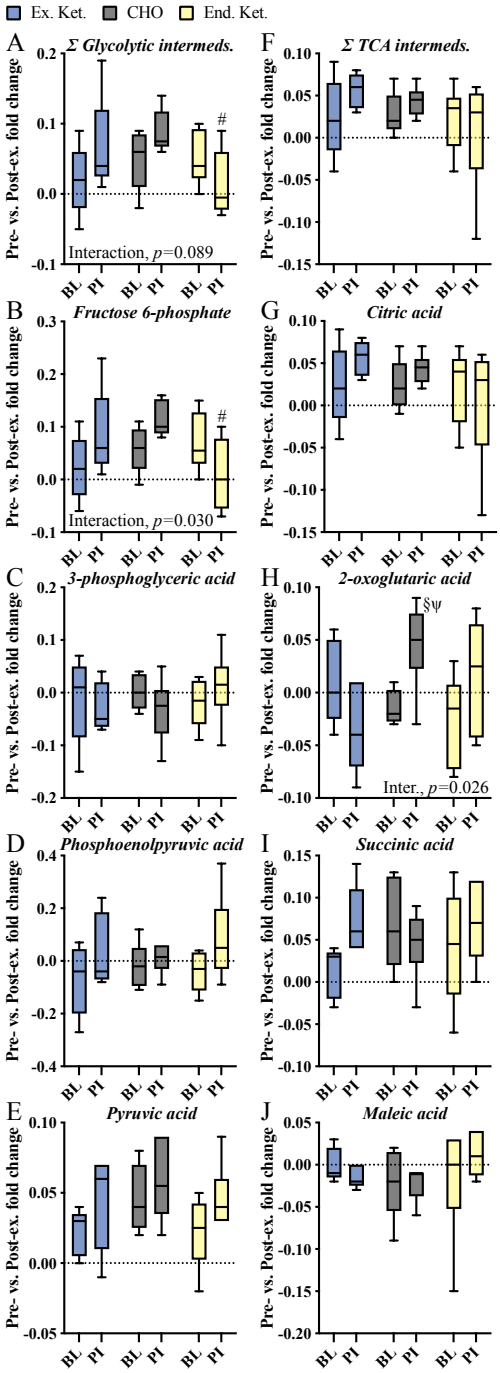


Figure 5.15: **Intramuscular glycolytic and TCA cycle metabolites.** Comparison of glycolytic **(A - E)** and TCA cycle **(F - J)** metabolite fold-changes at the baseline (BL) and post-intervention (PI) exercise tests. Between group comparisons of metabolite fold-changes were investigated by 2-way RM ANOVA. Significant post-hoc comparisons: Ψ = within group, post-intervention versus baseline; # = CHO versus endogenous ketosis groups at the equivalent visit. § = CHO versus exogenous ketosis at the equivalent visit. Data presented are log(10) transformed arbitrary units.

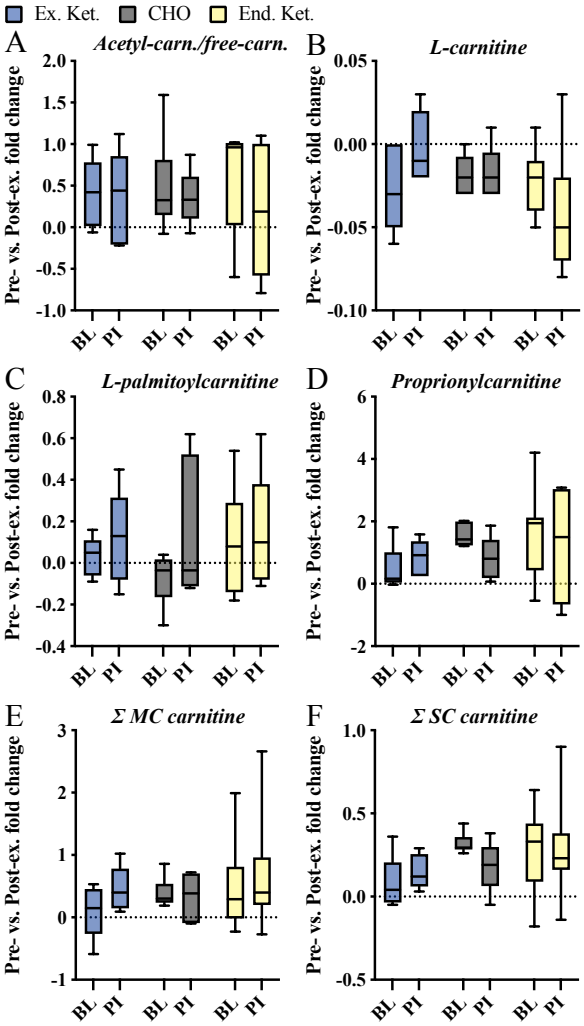


Figure 5.16: **Intramuscular carnitine derivatives.** Comparison of carnitine derivative fold-changes at the baseline (BL) and post-intervention (PI) exercise tests. Between group comparisons of metabolite fold-changes were investigated by 2-way RM ANOVA. Data presented are log(10) transformed arbitrary units (excluding ratios).

Ratings of perceived exertion

There was a significant main effect of condition for delta RPE (p=0.001), which was significantly greater in the endogenous ketosis versus exogenous ketosis (p=0.002) and CHO (p=0.006) conditions (Figure 5.19 B) during steady state exercise.

5.3.9 Muscle protein abundance

To gain insights into the molecular mechanisms that might underlie observed changes in both resting glucose metabolism (exogenous ketosis) and exercise metabolism

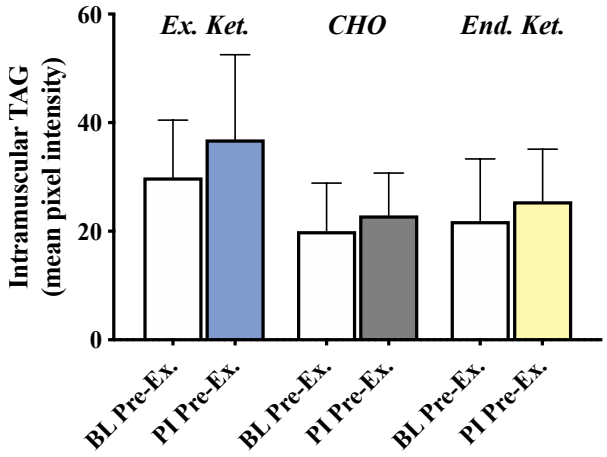


Figure 5.17: **Intramuscular triglyceride content measured in pre-exercise muscle biopsy samples.** Between group comparisons were investigated by 2-way RM ANOVA.

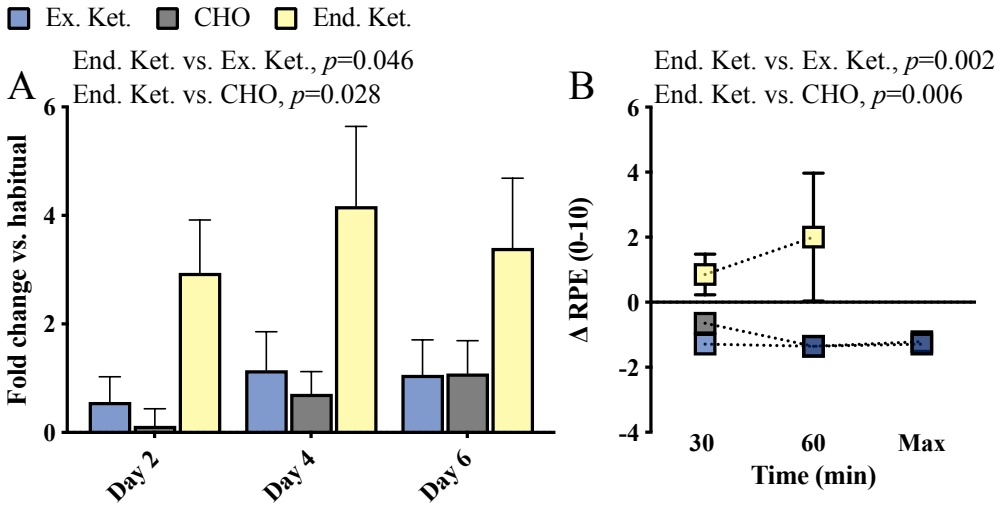


Figure 5.18: **Mood state and perceived exercise exertion. (A)** Fold changes (versus habitual scores recorded before the study) in the number of "worse than normal" responses to the DALDA questionnaire reflecting increased mood disturbance. Between group comparisons were investigated by 2-way RM ANOVA. **(B)** Delta RPE (post-intervention exercise test values - baseline exercise test values). t = 30 min and 60 min values were analysed via 2-way RM ANOVA using data from all three dietary conditions. Max values for exogenous ketosis and CHO conditions were analysed separately by Student's t-test.

(endogenous ketosis), quantitative, non-targeted (that is, label-free) proteomics was performed in vastus lateralis biopsy samples. Delta abundance values (representing post-intervention - baseline abundance) identified seven proteins that were differentially expressed between groups (Tables 5.3). The combination of ketogenic diet and vigorous exercise training caused significant relative increases in the expression of

carnitine O-acetyltransferase (catalyses the transfer of acyl groups to and from coenzyme A (CoA)) and carnitine/acylcarnitine translocase (transports carnitine and carnitine-fatty acyl complexes across the mitochondrial membrane). BDH expression - the catalyst for the first reaction of ketolysis (see Figure 1.2) - was significantly, relatively increased in the endogenous ketosis, but not the exogenous ketosis condition. The largest effect was observed in pyruvate dehydrogenase kinase 4 (PDK4) expression, which was ≈6-fold greater versus baseline in the endogenous ketosis condition. Expression of the two key regulatory enzymes of glycolysis - PFK and PDH - were also increased in the endogenous ketosis versus CHO condition. Thus, the expression of rate-limiting enzymes/transporters of three major metabolic pathways - glycolysis, lipolysis and ketolysis - were augmented by adoption of a ketogenic diet combined with vigorous exercise training. Interestingly, PDH expression was also significantly increased in the exogenous ketosis versus CHO group. However, no other differences in protein expression were observed between these conditions.

5.3.10 Adipocyte gene expression

The transcription of genes that regulate adipocyte cAMP signalling, lipolysis and glucose uptake were analysed in subcutaneous adipose tissue biopsy samples taken at the baseline and post-intervention exercise tests. The only difference observed was for GLUT 4, where expression was significantly greater versus baseline in athletes who undertook the ketogenic diet (p=0.031, Figure 5.19 A).

5.4 Discussion

This work sought to compare the effects of two distinctly different ketotic states, an exogenously- and an endogenously-induced ketosis, in well-trained athletes during a 10-day training intervention. The main findings were: (i) supplementing a habitual diet with a ketone monoester and CHO containing drink caused insulin resistance; (ii) adoption of a ketogenic diet caused a significant reduction in CHO utilisation

Table 5.3: **Significant intramuscular protein fold-changes.** Between group comparisons of delta protein abundance values (post-intervention values - baseline values) were initially assessed by 1-way ANOVA using a permutation-based FDR threshold of 0.05 with 250 randomisations applied. Explorative between-group post-hoc comparisons were then performed with the same FDR corrections applied. Significant post-hoc comparisons (highlighted in gray): # = endogenous ketosis versus CHO. * = endogenous versus exogenous ketosis. § = exogenous ketosis versus CHO. Data presented are mean ± SD.

	Ex. Ket.	**CHO**	**End. Ket.**	**ANOVA**	
	Fold Change	*Fold Change*	*Fold Change*	*-Log10 p value*	*q-value*
Pyruvate dehydrogenase kinase 4	0.145 ± 0.934	0.293 ± 0.810	5.916 ± 3.381$^{\#*}$	4.588	0.000
Pyruvate dehydrogenase complex	0.419 ± 0.738§	-0.012 ± 0.143	0.387 ± 0.442$^{\#}$	2.180	0.152
Carnitine/acylcarnitine carrier protein	-0.203 ± 0.175	-0.076 ± 0.433	1.121 ± 1.865$^{\#*}$	1.971	0.196
6-phosphofructokinase	0.072 ± 0.527	-0.092 ± 0.885	0.371 ± 0.646$^{\#}$	1.668	0.251
3-hydroxyisobutyrate dehydrogenase	-0.056 ± 0.306	-0.052 ± 0.262	0.813 ± 1.265$^{\#*}$	1.718	0.253
Iso. 2 of Carnitine O-acetyltransferase	0.003 ± 0.740	-0.133 ± 0.248	0.621 ± 0.583$^{\#*}$	1.731	0.294
Iso. 3 of Peroxisomal carnitine O-acetyltransferase	-0.086 ± 0.324	-0.023 ± 0.260	0.728 ± 0.898*	1.257	0.471

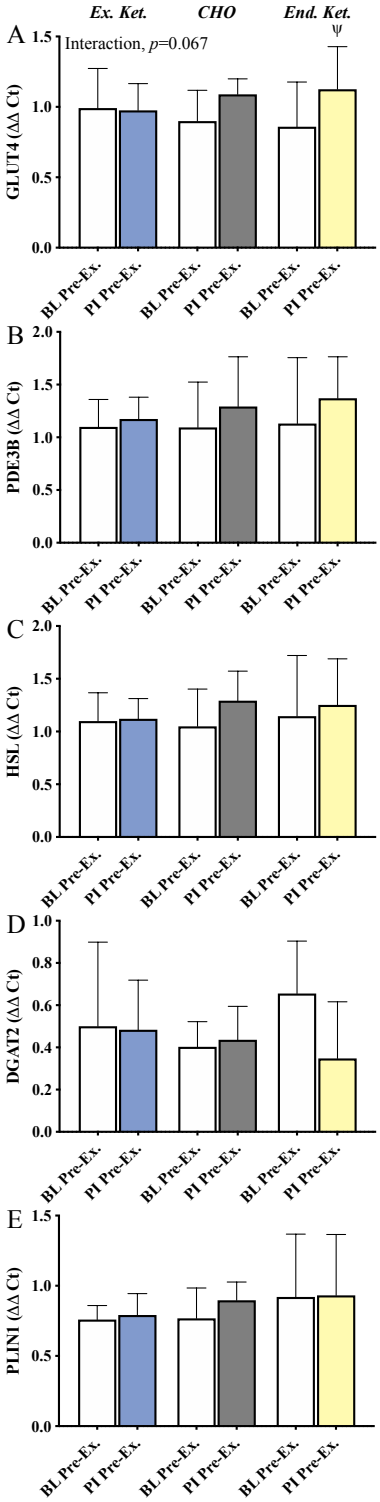


Figure 5.19: **Transcript expression of genes in adipocyte cAMP signalling, lipolysis and glucose transport.** Transcript expression of **(A)** GLUT 4, **(B)** PDE3B, **(C)** HSL, **(D)** DGAT2 and **(E)** PLIN1 in baseline (BL) and post-intervention (PI) adipocyte biopsy samples. Between group comparisons of delta-delta-C_t values were investigated by 2-way RM ANOVA. Significant post-hoc comparisons: Ψ = post-intervention versus baseline at the indicated measurement.

during exercise that, in part, was mediated by changes in muscle protein content (that is, muscle "retooling"). However, the reciprocal increase in fat oxidation was not sufficient to sustain exercise capacity at 70% of $VO_{2\ peak}$; and (iii) exercise capacity was significantly augmented by βHB supplementation - both versus the endogenous ketosis condition and versus individuals supplemented with an isocaloric CHO only drink.

5.4.1 A 10-day exogenously-induced ketosis and vigorous exercise training causes insulin resistance

The development of glucose intolerance in response to starvation or a very low CHO content diet has been known since the mid 19^{th} century [245]. This is typically attributed to an elevation in blood FFA concentration [246–248]. However, findings presented here indicate that a prolonged exposure to βHB, independent from elevated FFAs or the general metabolic milieu required to stimulate hepatic ketogenesis, may induce insulin resistance. Whilst the insulin resistance Ketosis is an evolutionary adaptation to sustain life during periods of food deprivation, primarily by reducing the rate of glucose utilisation in peripheral tissues [47, 55]. Thus, that βHB inhibits glucose uptake is consistent with its teleological purpose during starvation. Insulin resistance is a useful metabolic adaptation to food scarcity [249]. It is advantageous to limit the supply of finite glucose to peripheral tissues that can adequately meet energy requirements during starvation through the oxidation of fatty acids and, to a lesser extent, ketones [19, 57, 250]. Glucose can then be preferentially directed to those tissues that are largely reliant upon it, most notably the brain [47, 55]. Glucose transport across the blood-brain barrier primarily occurs in an insulin-independent manner via solute carrier family 2, facilitated glucose transporter member 1 (GLUT 1) [251] and as such, would not be impeded by short periods of insulin resistance. However, in modern Western societies ketosis is more commonly observed as a *consequence* of insulin resistance [252]. In this setting, ketone mediated glucose intolerance would be maladaptive, potentially serving to

perpetuate the pathological insulin resistant state.

Whilst the present study is the first to report that a prolonged exogenously-induced ketosis may inhibit glucose uptake in humans, previous work has demonstrated similar effects following the incubation of adult cardiomyocytes and isolated mouse soleus muscle in βHB [236, 237]. Notably, Yamada and colleagues observed that βHB inhibition of glucose uptake was apparent in soleus (slow-oxidative phenotype) but not extensor digitorum longus (fast/fast-glycolytic phenotype) mouse muscle (see Appendix H for a comparison of the proportion of slow-oxidative fibres in these tissues). Although not measured, the well-trained endurance athletes within this study are likely to have a high proportion of slow-oxidative muscle fibres relative to non-athletic populations [253]. It is plausible, therefore, that the effects of a prolonged exogenously-induced ketosis on insulin-stimulated glucose uptake would be less pronounced in non-athletic populations. Work is underway to assess this.

How a prolonged exogenously-induced ketosis causes insulin resistance is unclear; however, data presented here may rule out several potential mechanisms. Participants' blood βHB concentrations were negligible ($\approx$0.2 mM) during the OGTTs in the exogenous ketosis condition. As such, the effects on glucose uptake cannot be attributed to substrate-level inhibition (as described by the Randle cycle [14]), which is the prevailing hypothesis for the observed inhibition of glycolysis during ketosis [48, 71]. This suggests a model of metabolic/physiological adaptation that may include the synthesis and degradation of proteins to "retool" target tissues towards a reduction in glucose utilisation. Such a model could explain why long βHB incubation periods (>9.5 hours) were necessary for the inhibition of glucose uptake in cardiomyocytes [237] and isolated mouse soleus [236]. Furthermore, this would provide a rationale for the seemingly paradoxical findings that acute exogenously-induced ketosis may enhance glucose uptake [91] (although the mechanisms for this are unclear; see paragraph 1.3.2) whereas a prolonged exogenously-induced ketosis impairs it (as reported here). This hypothesis will be discussed in relation to three pivotal, insulin-sensitive tissues: skeletal muscle, adipose tissue and the liver.

Skeletal muscle

Skeletal muscle accounts for ≈80% of insulin-stimulated glucose disposal [254]. As such, this tissue represents a potentially good target for investigating any metabolic and/or physiological adaptations that could explain the observed insulin resistance in the exogenous ketosis condition. Proteomic analysis of vastus lateralis muscle samples showed a small but significant relative increase in the expression of PDH versus the CHO condition. A plausible explanation is that the increased expression of PDH reflects a compensatory mechanism for insulin resistance; that is, to maintain glycolytic flux, despite decreased insulin stimulated glucose uptake. This may have been particularly important given the vigorous exercise training. Overall, though, protein expression was largely unchanged in the exogenous ketosis condition, suggesting that ketone mediated skeletal muscle "retooling" was not the cause of insulin resistance.

A small aliquot of ^{13}C glucose was included in drink preparations at the OGTTs for a random subsample of participants in the exogenous ketosis and CHO groups (n=3 per group). The subsequent enrichment of respired gases (measured as the TTR) reflects the uptake *and* oxidation of glucose, predominately in skeletal muscle [254], and thus provides a complimentary measure of glucose tolerance [240]. Resting glucose oxidation was markedly enhanced at the post-intervention OGTT in the CHO condition - a finding that is consistent with previous reports of increased glucose uptake and enhanced metabolic flexibility following exercise training [255]. Conversely, glucose oxidation was lower at $t = 120$ min only at the post-intervention versus baseline OGTT in the exogenous ketosis condition. Given the substantial decrease in glucose uptake (discussed above), glucose oxidation was, therefore, surprisingly static. Indeed, the change in glucose oxidation (that is, post-intervention - baseline TTR values) explained just ≈24% of the change in blood glucose concentrations during the OGTTs. A possible explanation is that the capacity of skeletal muscle to oxidise glucose may have been increased, either in response to the exercise training (as observed in the CHO condition) and/or to to compensate for

insulin resistance (see Figure 5.20 for an illustration of this hypothesis). This hypothesis is supported by the relative increase in PDH expression versus the CHO condition (described above) and the similarity in substrate metabolism (proportion of fat and CHO oxidised) between CHO and exogenous ketosis conditions at the post-intervention exercise test (discussed later).

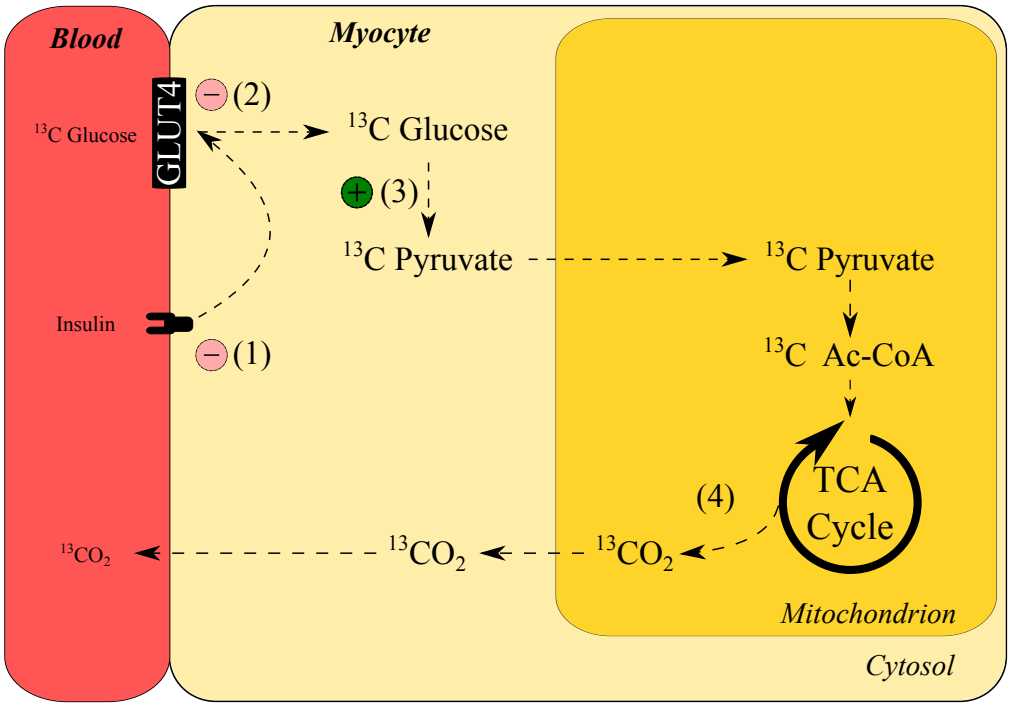


Figure 5.20: **A compensatory increase in glucose oxidation in the exogenous ketosis condition? (1)** Insulin signalling was impaired following the exogenous ketosis intervention, as observed by increases in blood glucose and insulin curves in response to a 75 g oral glucose load. **(2)** Consequently, GLUT 4 translocation to the cell membrane is likely to have been impaired, thus decreasing glucose uptake. **(3)** Given the substantial reduction in glucose uptake observed in the exogenous ketosis condition at the post-intervention OGTT, less intracellular glucose was available for oxidation. Despite this, glucose oxidation was relatively stable (indicated by only small decreases in the respiratory gas TTR at $t = 120$ min). As such, a compensatory increase in glycolytic capacity may have occurred. In other words, most likely due to metabolic adaptations made in response to exercise training [255] (as observed in the CHO condition), glucose oxidation rates were maintained, despite reduced glucose availability. **(4)** Consequently, the amount of ^{13}C-labelled CO_2 produced in the TCA cycle was similar at baseline and post-intervention OGTTs.

Adipose tissue

Drawing parallels to another endogenous inhibitor of adipocyte lipolysis, niacin (vitamin B3), may provide clues regarding the aetiology of the observed insulin resistance in the exogenous ketosis condition. As with βHB, the acute administration of niacin substantially lowers blood FFA concentrations through activation of HCAR2 and a subsequent decrease in intracellular cAMP signalling [93, 256]. Adipocytes appear to counteract this signal by altering gene expression in a manner conducive with increased lipolysis. Prolonged niacin administration causes: reduced expression of PDE3B [243, 257], an enzyme that degrades intramuscular cAMP in response to elevated blood insulin levels and as such, is pivotal to the antilipolytic effect of insulin [256]; increased expression of HSL [243], which catalyses the hydrolysis of triglycerides [258]; decreased expression of PLIN1 [243], an enzyme that coats lipid droplets and subsequently suppresses lipolysis by reducing lipase access to triglycerides [259]; and decreased expression of DGAT2 [243], which catalyses the final step of triglyceride formation [259]. The decrease in PDE3B is believed to be responsible for the adipocyte insulin resistance that occurs following prolonged administration of niacin [243, 257]. Collectively, these altered expression patterns may explain the "fatty acid rebound" phenomenon, whereby blood FFA concentrations rise above baseline levels $\approx$1 - 2 hours after administration of niacin [243]. An increase in circulating lipids could cause an accumulation of triglycerides in non-fat-storing tissues including muscle and liver (ectopic fat deposition) [243, 260], which is important to the pathogenesis of whole-body insulin resistance [261].

Given the joint actions of βHB and niacin on the HCAR2 receptor, it is plausible that a similar signalling cascade might have caused the observed insulin resistance in the exogenous ketosis condition. However, PDE3B, HSL, PLIN1 and DGAT2 expression were unchanged, nor was there a difference in GLUT 4 expression, which is a strong marker of insulin resistance [262]. Additionally, intramuscular triglyceride concentration was similar in post-intervention versus baseline muscle samples in all groups. Taken together, it appears unlikely that adipocyte insulin resistance

or ectopic fat deposition in skeletal muscle can account for the observed insulin resistance in the exogenous ketosis condition.

Liver

The liver plays a central role in the maintenance of systemic glucose homeostasis and aberrant hepatic insulin action is thought to be a primary driver of whole-body insulin resistance [263]. Indeed, the glucose intolerance commonly observed following adoption of a ketogenic diet has been attributed to selective hepatic insulin resistance, rather than impaired muscle or adipose tissue glucose uptake [234]. An insulin resistant liver produces excess glucose, which in turn stimulates insulin secretion causing mild hyperglycaemia and hyperinsulinaemia. In the present study, fasting blood glucose concentration increased from 5.88 ± 1.17 mM to 6.43 ± 1.21 mM; however, no differences in fasting blood insulin or in the HOMA IR - a well validated method to assess hepatic insulin resistance and β cell function [264] - were observed. It is plausible that an elevation in hyperglycaemia may precede hyperinsulinaemia and, therefore, had the study duration been longer both fasting insulin and the HOMA IR would also have been elevated. However, recent work by Soto-Mota and colleagues [149] found that supplementing healthy individuals with 25 mL of the ketone monoester three times daily for 28 days had no effect on fasting glucose, HOMA IR, glycated Hb or lipid profiles. Nonetheless, further work is warranted to determine the effects of prolonged ketone consumption on the liver.

If prolonged exposure to ketones causes insulin resistance, a pertinent question is why glucose tolerance was unaffected in the endogenous ketosis condition. It is plausible that other biochemical, hormonal, transcriptional or posttranslational adaptations to a ketogenic diet and vigorous exercise training compensated for any insulin desensitising effects of ketones. Evidence for this is provided by the increased expression of GLUT 4 in adipocytes, which may provide protection against high-fat-diet-induced insulin resistance [265]. Subjects in the endogenous ketosis condition did lose a significant amount of weight, which may be insulin-sensitising

[266]. However, ≈60% of this weight loss occurred on the first study day suggesting it was mostly water mass [239]. Moreover, it seems unlikely that an ≈4% (≈3 kg) change in body weight would have a substantial effect on insulin sensitivity in this cohort of well-trained athletes.

In sum, findings presented here raise several important questions, including but not limited to: (i) whether the effects on insulin sensitivity are also observed in healthy, non-athletic individuals; (ii) how quickly glucose intolerance manifests following ketone monoester supplementation; (iii) how quickly insulin sensitivity returns to baseline following cessation of ketone monoester ingestion; and (iv) what is the aetiology of the insulin resistance. Addressing these questions may provide novel insights into insulin resistance and its associated pathologies.

5.4.2 An endogenously-induced ketosis for exercise training?

The present work demonstrates that adopting a short-term ketogenic diet during vigorous exercise training augments potentially advantageous adaptations that appear to primarily increase the capacity of muscle to utilise fat-derived substrates for energy production. This included an increased expression of mitochondrial fatty acid transport proteins - carnitine O-acetyltransferase and the carnitine/acylcarnitine carrier protein - which represent a bottleneck in the rate of fatty acid oxidation [26]. Most strikingly, there was a 6-fold increase in PDK4 expression, which has a pivotal role in suppressing glycolytic flux through its inhibitory effect on PDH [267]. Notably, BDH expression, the catalyst for the first reaction of ketolysis when βHB is the starting substrate (see Figure 1.2), was increased in the endogenous, but not exogenous ketosis condition. Thus, elevating blood ketones per se is not sufficient to increase skeletal muscles' capacity for ketone oxidation. At least in part, these protein-level adaptations contributed to the ≈60% increase in fat oxidation ($g \cdot min^{-1}$) at the post-intervention exercise test in the endogenous ketosis condition. However, we cannot discount that this simply reflected a reduction in glycogen content, which

was not determined in the present study (see limitations). A novel finding of the present work is that a short-term ketogenic diet and vigorous exercise training also increased the relative (to the CHO condition) expression of the key regulatory enzymes of glycolysis: PFK and PDH. This somewhat counter-intuitive finding may reflect the conflicting environmental adaptive signals: a dietary signal expected to stimulate adaptations to increase fat oxidation [99, 108, 268], and an exercise signal, which might have promoted adaptations that increase muscles' capacity to utilise limited CHO stores in order to meet the high energy demands of the physical activity undertaken (as observed in the CHO condition, see Figure 5.8).

The increased capacity for FFA oxidation, reaching a maximum fat oxidation rate of 1.17 $g \cdot min^{-1}$, was not sufficient to maintain exercise at an intensity relevant to the majority of competitive endurance events (that is, $\geq$70% W_{Max}). Indeed, just one athlete was able to complete the 90 min steady state exercise following adoption of the ketogenic diet, which they achieved on three occasions. Additionally, both perceptions of exercise exertion and physiological markers of exercise effort (VO_2, V_E and HR) were elevated during exercise at 70% $VO_{2\ peak}$. Furthermore, athletes in the endogenous ketosis condition had markedly increased mood disturbance, consistent with non-functional overreaching [269]. Proponents of the ketogenic diet suggest adaptation periods in excess of two weeks are required to achieve "fat or keto adaptation" and realise the potential performance benefits [99, 108, 268]. It is possible, therefore, that had the study intervention period been longer, further adaptations may have occurred and exercise capacity would have been normalised or improved. However, it is notable that the fat oxidation rates reported here are similar to those previously reported following a four to six week ketogenic diet in athletes ($\approx$1.5 $g \cdot min^{-1}$ [100, 103]). No quantifiable definition of "fat or keto adaptation" exists. Further work is warranted to explore the breadth of physiological and metabolic adaptations that may occur following a ketogenic diet, perhaps best achieved through a multi-omics approach, and the temporal nature of these.

5.4.3 An exogenously-induced ketosis for acute exercise performance?

In contrast to the endogenous ketosis condition, exercise metabolism was largely unaffected in athletes supplemented with ketones. Blood substrate, intramuscular metabolites and cardiorespiratory measures were similar to those in the CHO condition. One notable exception was V_E, which was significantly elevated versus the CHO condition at $t = 30$ min, potentially reflecting a physiological adaptation to exercise training during ketoacidaemia [121]. In the overnight fasted state (as performed here), circulating glucose is a substantial (≈20%) contributor to overall energy expenditure during cycling exercise at 75% W_{Max} [18]. Whilst *resting* insulin-stimulated glucose uptake was significantly impaired in the exogenous ketosis condition, exercise causes GLUT 4 translocation in an insulin-independent manner [270] (an important factor in the insulin-sensitising effects of exercise [244]). In part, this may explain why the marked changes in resting glucose tolerance did not affect exercise substrate metabolism. However, it is plausible that the effects of prolonged ketone monoester ingestion on glucose uptake could impede exogenous CHO oxidation rates during exercise, with potentially negative implications for exercise capacity/performance.

Exercise capacity was significantly enhanced in the exogenous ketosis condition; both versus the endogenous ketosis and, of greater relevance, versus the CHO condition also. Indeed, athletes supplemented with a ketone monoester and CHO drink outperformed their counterparts receiving an isocaloric amount of CHO on 50% of training intervention days (days 1, 4 and 5), with no significant differences observed between the groups on the remaining days. Unlike the previous report of enhanced exercise capacity by our laboratory [71], athletes were permitted to eat pre-exercise meals (that is, exercise was performed in the postprandial state), thus enhancing the "real-world" applicability of these findings. Given that exogenous βHB contributes minimally to overall energy expenditure (as discussed in Chapter

4), the most plausible explanation for the observed increases in exercise capacity is the glycogen "sparing" (or impairing) effect observed during an exogenously-induced ketosis [71, 106]. However, recent work has questioned this effect, finding that intramuscular glycogen content was not different following 2 hours of race simulation cycling exercise when athletes were supplemented with CHO (60 $g \cdot h^{-1}$) plus a ketone monoester drink or minimal-calorie control drink [123]. To help elucidate the mechanisms through which ketones may enhance exercise capacity, direct measures of fat and CHO oxidation using stable isotope techniques are warranted.

5.4.4 Limitations and future directions

To the author's knowledge, this is the first study to use an untargeted proteomics approach to investigate skeletal muscle adaptations to exercise training. The relatively high participant attrition rate (16%) and the impracticality of enrolling large numbers of athletes meant proteomic analyses were likely underpowered, thus increasing type II error. Accordingly, the protein fold-changes reported here likely represent the minimum adaptations achieved.

Groups were well matched for physiological and anthropometric measures. However, there was an uneven gender distribution, with just one female completing the study (enrolled to the exogenous ketosis condition). Participant data were adjusted to baseline values (that is, the difference (delta) between post-intervention and baseline values were compared between groups). Therefore, in essence, each participant acted as their own control. As such, the gender disparity is unlikely to have influenced outcomes. Nonetheless, it is important to recognise the presence of sexual dimorphism in human metabolism [271].

Fasted glucose levels were unusually high both pre- and post-intervention, particularly given the well above population average physical activity levels of the participants recruited. It is believed that the apparatus used to measure blood glucose concentrations may have introduced a systematic bias to the reported values.

Intramuscular metabolites were measured in muscle biopsy samples taken at

maximal-intensity exercise (that is, immediately upon cessation of exercise). On reflection, performing muscle biopsies immediately after the 70% $VO_{2\ peak}$ exercise - an intensity where muscle is not wholly reliant on CHO [18] - would have increased the likelihood of determining potentially subtle but important differences in intramuscular metabolites. Furthermore, the greater-than-expected decrease in exercise capacity restricted comparisons between baseline and post-intervention exercise metabolism in the endogenous ketosis condition. Taken together, it may have been advisable to eliminate the incremental-intensity exercise component at the baseline and post-intervention exercise test visits, and to have reduced the steady state exercise duration to 60 min.

An important outstanding question is whether supplementing individuals post-exercise with a ketone monoester drink + CHO can augment intramuscular glycogen synthesis [107, 121, 136]. To address this, sectioned muscle samples were stained with periodic acid-Schiff and imaged using a slide scanner [71, 272]. However, the consistency of staining and image quality was deemed insufficient to accurately determine differences in intramuscular glycogen content and as such, this planned analysis was not performed.

5.5 Conclusions

Supplementing athletes with a βHB and CHO drink for 10 days during strenuous exercise training markedly reduces insulin stimulated glucose uptake but does not alter exercise metabolism. Furthermore, exercise capacity is significantly enhanced when compared to athletes supplemented with an isocaloric CHO only drink. Conversely, elevating blood ketones through adoption of a ketogenic diet causes a substantial increase in fat oxidation during exercise that, at least in part, is mediated by protein-level adaptations in skeletal muscle. However, exercise capacity during a ketogenic diet is substantially impaired. These findings demonstrate the distinct differences between a physiological, dietary-induced ketosis, and the unique physiological state of an exogenously-induced ketosis.

Chapter 6

General discussion

6.1 Main findings

- Athletes compensate for a mild ketoacidaemia during incremental-intensity exercise through an ≈5% increase in total V_E.
- Exercise-induced hyperventilation (T_{Vent}) occurs at an ≈20 W lower workload, with an approximately equal-in-magnitude and opposite-in-direction increase in power at the FBLA of 4 mM. Consequently, blood lactate accumulation is dissociated from the T_{Vent} during an exogenously-induced ketosis.
- Athletes supplemented with βHB have increased perceptions of leg discomfort and anxiety related to leg discomfort and breathing during incremental-intensity exercise. This effect is primarily driven by ketoacidaemia (that is, an elevated blood H^+ concentration).
- Increasing blood βHB concentration from ≈2 mM to ≈4.5 mM has no effect on exogenous βHB oxidation rates, nor ketone signalling actions (observed as changes in blood substrate concentrations during ketosis) at rest or during incremental-intensity cycling exercise.
- Exogenous βHB oxidation rate peaks at 0.1 $g \cdot min^{-1}$ during moderate-to-hard-intensity (50% and 75% W_{Max}) cycling exercise.
- The relative contribution of exogenous βHB oxidation to overall energy expenditure is small (≈2.5% to ≈4.5%) and peaks during very-light-intensity

exercise.

- Delta efficiency is increased ≈7% during cycling exercise at a blood βHB concentration of ≈2 mM during an exogenously-induced ketosis.
- Skeletal muscle phenotype may be an important predictor of exogenous βHB oxidation rates in athletes.
- Supplementing athletes with a ketone monoester + CHO drink versus an isocaloric CHO-only drink before and during exercise may enhance physical capacity by between 4% and 6%.
- Conversely, athletes who adopt a ketogenic diet and are supplemented with a fat containing drink before and during exercise have impaired exercise capacity of between 41% and 48% versus a CHO control group.
- Adopting a 10-day ketogenic diet during vigorous exercise training markedly increases fat oxidation (from 0.42 ± 0.18 g·min^{-1} to 1.17 ± 0.41 g·min^{-1}) during cycling exercise. In part, this was mediated by protein-level adaptations in skeletal muscle.
- Conversely, supplementing a habitual diet with a drink containing ketone monoester and CHO for 10 days during vigorous exercise training caused insulin resistance, without affecting CHO and fat metabolism during exercise.

The implications of these findings for the use of exogenous βHB in exercise performance and as a therapeutic agent, together with directions for future research in these areas, are now discussed.

6.2 The use of an exogenously-induced ketosis for exercise performance

Despite reported widespread adoption within elite sport [273], optimal usage guidelines for ketone supplements have not been determined. The following section dis-

cusses how the findings of this Thesis may inform these.

6.2.1 Dosing

A blood βHB concentration threshold of $\approx \leq$2 mM was demonstrated in Chapter 3, both for exogenous βHB oxidation and ketone signalling (the latter being reflected by changes in blood glucose, NEFA and lactate). Thus, the potentially advantageous effects of an exogenously-induced ketosis on exercise metabolism are likely achieved at low doses of the ketone monoester. In athletic cohorts, doses of between 135 and 187 mg·kg BW^{-1} (achieving a blood ketone range of ≈1.5 - ≈2 mM) are likely optimal. Indeed, given the dose-dependent perturbations to acid-base balance observed in Chapter 4, exercise undertaken with blood βHB concentrations ≈>2 mM may nullify any ergogenic effects, or could even be deleterious. This is supported by recent work demonstrating that during ≈3 h 15 min of intermittent-intensity cycling exercise, supplementing athletes with a ketone monoester alone (blood βHB = ≈2 - ≈3 mM) had no effect on performance but the combination of ketone monoester with sodium bicarbonate ($NaHCO_3$; thus increasing the blood buffering capacity) resulted in a significant 5% improvement [126]. Work presented in Chapter 3 [121] suggests that, in part, this may be attributed to a greater awareness of exercise exertion during a mild ketoacidaemia.

6.2.2 Athlete phenotype

In Chapter 3, preliminary evidence is presented demonstrating a positive correlation between the percentage of slow-oxidative fibres in vastus lateralis muscle and exogenous βHB oxidation rates. This finding is consistent with the previous observation that BDH and SCOT (the first two enzymes of ketolysis, see Figure 1.2) activity are ≈2.5 times and ≈1.5 times greater in slow-oxidative versus fast-glycolytic fibres [60]. Whilst these preliminary findings must be substantiated, they suggest that endurance athletes, who are well-observed to have a remarkably high proportion of slow-oxidative fibres [274–276], may benefit most from ketone supplementation.

Indeed, given the accompanying acid load and potentially reduced metabolic clearance rates, supplementing power athletes with ketones may even be deleterious to exercise performance.

6.2.3 Optimum duration and intensity of exercise

Two mechanisms have been proposed to explain improvements in exercise capacity during an exogenously-induced ketosis [70, 71, 80, 106]: (i) exogenous ketones may provide an alternative, energy-efficient substrate for working skeletal muscle; and (ii) ketone metabolism may suppress glycolytic flux, resulting in a "rationing" of intramuscular glycogen whilst simultaneously increasing intramuscular triglyceride catabolism [106]. In Chapter 4, it was shown that the contribution of exogenous βHB oxidation to overall energy expenditure in exercising skeletal muscle is low versus CHO- and fat-derived substrates [18]. Furthermore, the greatest contribution ($\approx$4.5%) was at an intensity that is not relevant to exercise performance (25% W_{Max}). These findings imply that ketosis-mediated alterations in CHO and fat utilisation (that is, the second proposed mechanism) may underlie observed improvements in exercise performance (in Chapter 5 and previously reported [71, 126]). This has important implications regarding the types of exercise where ketone supplementation may be advantageous.

Exercise capacity is impaired when glycogen stores are depleted (commonly known to athletes of all levels as "hitting the wall") [33–35, 43]. Consequently, the ketosis-mediated "rationing" of glycogen and concomitant increase in intramuscular triglyceride catabolism may help sustain performance during prolonged, moderate-to-high-intensity endurance exercise. However, at very high exercise intensities (for example, race-winning sprints) lactic glycolysis contributes significantly to ATP resynthesis [3] (see Paragraph 1.1.5). In this setting, the inhibition of glycolysis observed following ketone supplementation may be detrimental to exercise capacity [80]. As such, it is highly likely that an exercise intensity/duration threshold exists, where ketone supplementation moves from being ergogenic to ergolytic. In

part, such a threshold would explain why ketone monoester supplementation has been shown to enhance physical capacity in exercise lasting ≥90 min (as reported in Chapter 5 ([250]) and elsewhere [71, 126]) but had no effect on exercise performance during high-intensity intermittent running [112] or during an incremental ramp test (lasting ≈40 min) on a cycling ergometer (as reported in Chapter 3 ([121]). Work is underway to determine whether this performance threshold exists and where it lies on the continuum of exercise intensity/duration. It should be noted that recent work found that supplementing athletes with CHO (60 $g \cdot h^{-1}$) with either a ketone monoester (60 g total) or placebo (minimal calorie) drink did not affect intramuscular glycogen content following ≈3 hours of variable intensity cycling [123]. Consequently, direct measures of CHO and fat oxidation rates in exercising humans during an exogenously-induced ketosis are warranted.

6.2.4 Training

The contents of an athlete's diet may modulate recovery from exercise training [228, 277]. Current nutritional guidelines focus on adequate CHO and protein intake, with the amounts and proportion of each depending on the athlete's training priorities [238]. Recent work has demonstrated that supplementing current "gold-standard" nutritional guidelines with βHB may augment glycogen synthesis [107] and muscle protein synthesis/repair [85, 132]. Additionally, supplementing athletes with βHB post exercise during a period of exhaustive exercise training may attenuate the risk of non-functional overreaching [136]. Thus, whilst further work is required to substantiate these findings, there appears to be a sound rationale for inducing a prolonged exogenously-induced ketosis in athletes, requiring the ingestion of repeated ketone monoester drinks. However, findings presented in Chapter 5 suggest this may be inadvisable. Supplementing athletes with βHB caused insulin resistance, observed as a 27% increase in blood glucose AUC in response to a drink containing 75 g glucose. Remarkably, this occurred within just 10 days of three-times-daily supplementation and during vigorous exercise training, which is insulin-sensitising [244].

Insulin resistance causes an altered molecular response to acute exercise, covering several biological processes, including inflammation, cardiovascular signalling, energy metabolism and metabolic hormones [278]. Whilst not observed in the present study, it is plausible that inducing even a mild insulin resistance by prolonged ketone monoester ingestion may, therefore, impair adaptive responses to exercise training. Moreover, this finding has clear ramifications regarding the long-term health of athletes. Thus, a research priority must be to substantiate this finding and attempt to determine the aetiology (discussed in depth in paragraph 5.4.1). Until this work is completed, it is the author's opinion that athletes do not frequently supplement their diet with ketones.

6.2.5 A "train low, race high" approach to augment exogenous ketone utilisation?

In order to "ramp-up" the adaptive signal to endurance exercise training without comprising exercise capacity/performance, sports physiologists and athletes have sought to implement a "train low, race high" approach to intramuscular glycogen content [230, 279]. Here, short-term adaptation to a ketogenic diet and exercise training (demonstrated by changes in intramuscular protein content in Chapter 5 and elsewhere [228]) is followed by glycogen restoration via consumption of a CHO-rich diet for one to three days [235, 280–282]. This dietary manipulation robustly re-tools muscle to increase fat oxidation rates without comprising the availability of glycogen to support high-intensity efforts [281, 282]. In Chapter 5, it was demonstrated that ketolytic enzyme expression is increased following adoption of a ketogenic diet. This may result in greater exogenous ketone oxidation rates, although work is warranted to test this hypothesis. As such, a similar "train low, race high" approach may be employed to prime muscle to utilise exogenous βHB during exercise, whilst ensuring plentiful glycogen.

6.3 Therapeutic application of an exogenously-induced ketosis

The profound metabolic effects of an exogenously-induced ketosis suggest it may have utility as an adjunctive treatment for a range of chronic disorders either caused or exacerbated by dysfunctional metabolism [17, 109, 146] (see Table 6.1 for a summary of proposed therapeutic effects in a selection of chronic disorders). This would necessitate that patients consume repeated doses of the ketone monoester drink. Findings presented in Chapter 5 suggest that any hypothesised therapeutic effects may be outweighed by the potential to induce (or exacerbate) insulin resistance. Indeed, insulin resistance is strongly associated with the aetiology of all the chronic conditions listed within Table 6.1 [283–285]. It is possible that the pharmacokinetics of the ketone monoester (that is, its absorption, distribution, metabolism and excretion) was affected by the exercise intervention in Chapter 5, as is case for several drug therapies [286]. Further work is warranted (and underway) to determine whether similar effects are observed in healthy, non-athletic individuals that are not undertaking repeated bouts of exhaustive exercise. Presently, however, using an exogenously-induced ketosis as an adjunctive treatment for chronic disorders appears inadvisable. Moreover, elucidating the mechanisms responsible for the observed insulin resistance may provide novel insights into the causes of dysfunctional CHO metabolism underlying much of the chronic diseases inflicting modern Western societies. In particular, it will be interesting to determine whether the insulin resistance is physiological or pathophysiological in nature. In other words, does it represent a potentially useful adaptation to states of depleted CHO availability (thus preserving glucose for the brain - see paragraph 5.4.1) or is it a deleterious response to the unique physiological state of an exogenously-induced ketosis.

Much of the enthusiasm regarding the use of exogenous ketones as an adjunctive treatment for chronic disorders is derived from the observed efficacy of ketogenic diets [98]. For example, adopting a ketogenic diet has beneficial effects on glycaemia

Table 6.1: **Summary of the proposed therapeutic benefits of an exogenously-induced ketosis in select chronic diseases**

Type II DM	Prominent metabolic features of type II diabetes melitus or insulin-resistant states are elevated blood glucose concentrations and dyslipidaemia. Both blood glucose and NEFA concentrations are markedly lowered during an exogenously-induced ketosis [71, 92]. However, there is currently no evidence this translates to long-term improvements in glycaemic control or blood lipid profiles [149]. Cardiovascular disease accounts for ≈60% of mortality in type II diabetics. Ketones appear to be a preferential fuel for the heart [58] and may improve cardiac energetics [69, 287, 288], suggesting ketone supplementation may be an adjunctive treatment to help prevent cardiac failure [109]
Cachexia	Muscle protein catabolism is decreased during a starvation-induced ketosis [19, 47] and exogenously-induced ketosis [71, 83, 85–87]. Additionally, βHB may reduce sympathetic tone (associated with catabolic processes [97]) through antagonism of FFAR3 [96].
Obesity	Consumption of a ketone monoester drink decreases hunger and increases plasma ghrelin concentrations versus an isocaloric CHO containing drink [289], suggesting it may be a useful adjunct to a weight loss program. However, consuming 25 g of a ketone monoester three times daily for 28 days had no effect on body mass [149].
PD	Parkinson's Disease (PD) is marked by deficits in mitochondrial metabolism, including reduced ATP synthesis - in part mediated by dysfunction of Complex I of the electron transport chain [290] - and increased reactive oxygen species production [291]. βHB oxidation may enhance mitochondrial metabolism by reducing oxidative stress [146], and by circumnavigating complex I dysfunction by increasing the supply of electrons to complex II via succinate [146, 292]. Accordingly, βHB has been shown to protect neurons in murine models of Parkinson's Disease [293].

[280–282, 294] and has been shown to reverse type 2 diabetes in ≈50% of patients at 2-year follow-up [295]. This, together with the well-observed decrease in blood glucose and NEFA concentration following ketone supplementation (in Chapters 3 and 4 and elsewhere [65, 92]), has led to suggestions that the beneficial effects of a ketogenic diet on glucose metabolism may result from a ketone-specific mechanism [109]. However, as demonstrated by work presented here, these two ketotic states have distinctly different effects on human metabolism and, in particular, on CHO metabolism.

Perhaps the most effective therapeutic application of an exogenously-induced ketosis will be the acute supplementation in patients with inborn errors of metabolism. Here, ketones may serve as an alternative metabolic substrate in genetic disorders of reduced glucose uptake/oxidation (for example, GLUT 1 deficiency, glycogen storage disorders, PDH deficiency) or reduced fatty acid uptake/oxidation (for example, very-long-chain acyl-CoA dehydrogenase deficiency, Acyl-CoA-dehydrogenase deficiencies or carnitine-acyl-carnitine translocase deficiency) [296]. Recent work has provided promising preliminary results demonstrating improvements in exercise metabolism in very-long-chain acyl-CoA deficient patients supplemented with the ketone monoester [297]. This may extend to neurological disorders marked by defects in mitochondrial metabolism (see the proposed therapeutic application of ketone supplementation in Parkinson's Disease in Table 6.1) [146]. In these settings, the acute supplementation of ketones may enhance physical capacity allowing them to undertake potentially therapeutic bouts of exercise training [298, 299], which may otherwise be prohibitively tiring. Indeed, preliminary findings shows that Parkinson's Disease patients have a striking 24% increase in cycling exercise capacity when supplemented with a CHO and ketone monoester drink versus an isocaloric CHO-only drink [300].

6.4 Concluding remarks

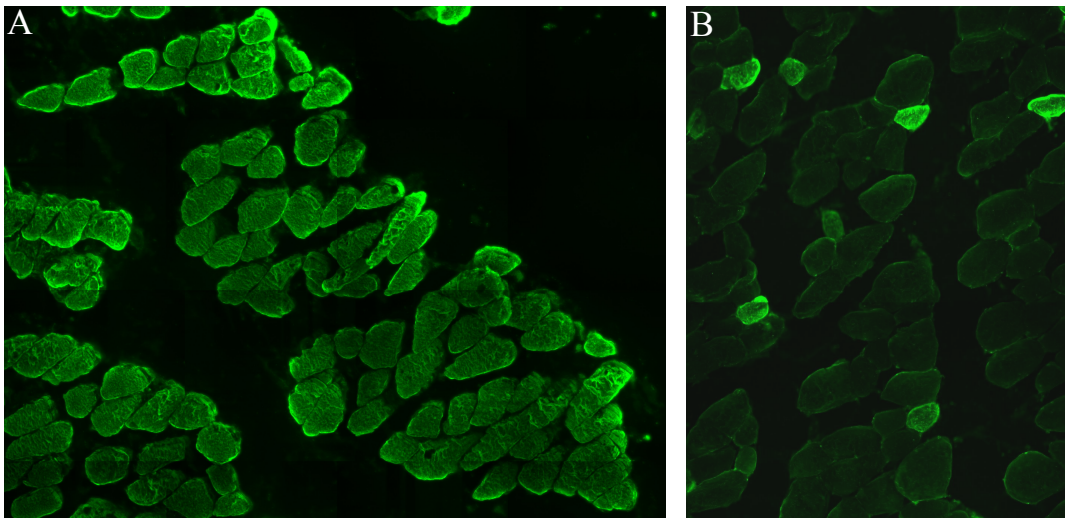
Work presented here demonstrates the profound effects of an exogenously-induced ketosis on human physiology and metabolism. Whilst our understanding of this novel physiological state is growing rapidly, much work is required to understand if and how it may be best applied to enhancing human physical capacity and health.

Chapter 7

Appendices

Appendix A

Validation of anti-slow skeletal myosin antibody



Validation of the Anti-slow skeletal myosin heavy chain (ab11083, Abcam, UK) primary antibody. Fluorescent green fibres represent a slow-oxidative phenotype. Validation was performed in: **(A)** rat soleus muscle (extremely high proportion of slow-oxidative fibres [156]); and **(B)** rat extensor digitorum longus (extremely low proportion of slow-oxidative fibres [156]). Rats were untreated animals being culled as part of a separate study [301].

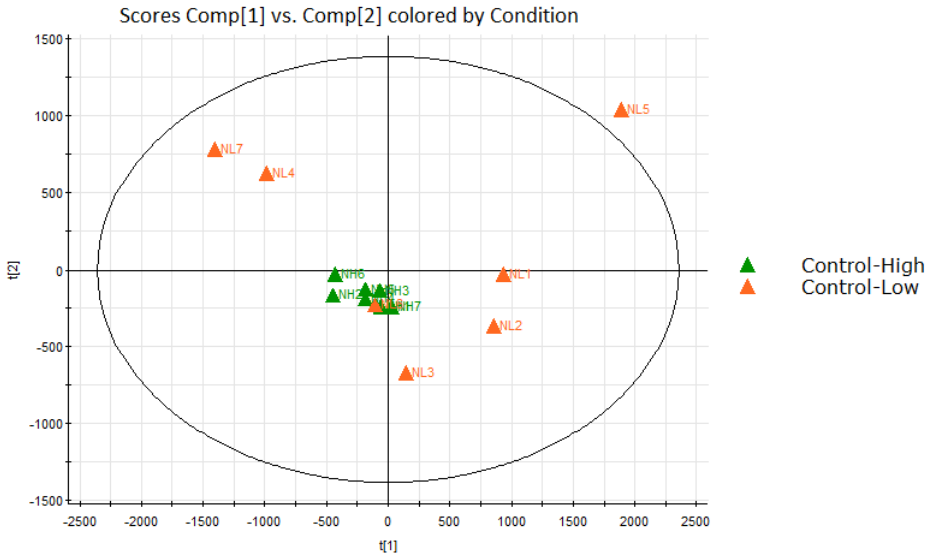
Appendix B

Metabolomics optimisation studies

Experiments were undertaken to optimise methods for metabolite extraction from skeletal muscle tissue samples, as part of the work undertaken in Chapter 5.

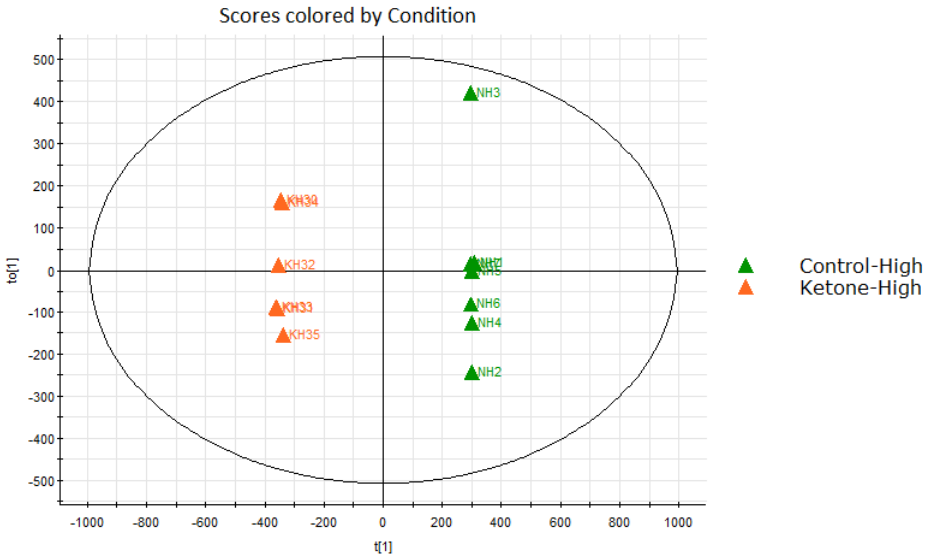
Optimisation experiment 1: In the first optimisation experiment, the effect of skeletal muscle sample size on the number and abundance of compounds detected was determined. "Small" (21.0 ± 4.3 mg; approximately equivalent to one human tissue sample) and "large" (46.6 ± 10.4 mg, approximately equivalent to two human tissue samples) skeletal muscle tissue samples from untreated (n=7) rats being culled as part of a separate experiment [301] were collected. Samples were homogenized in a methanol buffer, normalised to DNA concentration and analysed by ion exchange liquid chromatography-mass spectrometry. For both groups, a normal number of compounds were detected (≈100), and with low CV (<30%), indicating that the extraction protocol was robust. Given that samples were paired (that is, a small and large tissue sample was taken from each rat) and all rats were untreated, it was anticipated that measured metabolite abundances would be the same. However, differences were noted, particularly for those metabolites with a signal intensity <1000 and more commonly, <100 (see first Figure, below). Consequently, two human skeletal muscle tissue samples were used for metabolite analyses in Chapter 5 (average mass 38 ± 5.4 mg).

Optimisation experiment 2: The second optimisation study sought to determine whether expected differences in metabolite profiles could be determined between "large" skeletal muscle tissue samples taken from untreated (n=7; as per experiment 1) and streptozotocin-diabetic (n=7; 47.7 ± 7.5 mg) rats (being culled as part of the same experiment referenced in experiment 1 [301]). Two metabolically distinct groups were identified (see second Figure, below). As expected, diabetes resulted in an overall decrease in the concentration of glycolytic and TCA cycle metabolites in muscle. Thus, the number of samples (n=7 per group) and the metabolite extraction methods employed were adequate to determine expected differences in metabolite



OPLS-DA analysis: TCA method effect of biopsy size. Variance explained, (R^2Y(Cum) = 90%; variance predicted, Q^2(Cum) = 69%. Detected ions = 1482.

profiles in diabetic rats. Of course, it is possible that more modest differences in metabolite composition (than those expected with streptozotocin-diabetes) may require larger group sizes.



OPLS-DA analysis: TCA method effect of streptozotocin-diabetes. Variance explained, (R^2Y(Cum) = 99%; variance predicted, Q^2(Cum) = 88%.

Appendix C

Arterialised venous blood pH and bicarbonate conversion

Measured arterialised venous blood sample (attained using the heated hand technique [151]) pH and HCO_3^- values (denoted as venpH and venHCO_3^-) were converted into approximate arterial values. A "buffer line" was drawn through the venous pH and HCO_3^- values with a gradient of:

$$-(8.2+9.2 \cdot Hb) \tag{C.1}$$

The buffer line was then shifted to the left by a distance of:

$$0.1 \cdot (0.98 - \text{mixed venous oxygen saturation (SvO2)}) \tag{C.2}$$

This shift represents the increased saturation of Hb in arterialised blood and, therefore, reduced buffering capacity. This line is given by the equation:

$$HCO_3^- \text{ concentration} = -(8.2+9.2.Hb) \cdot pH + venHCO_3^- + \alpha \cdot venpH - \alpha \cdot 0.1 \cdot (0.98 - S_vO_2) \tag{C.3}$$

The intersection point between the second buffer line and the line given by the Henderson-Hasselbalch equation:

$$pH = pK + log10(HCO_3^- / (\alpha \cdot \text{partial pressure of carbon dioxide } (PCO_2))) \tag{C.4}$$

by rearranging equation C.5.

$$HCO_3^- \text{ concentration} = \alpha \cdot PCO_2 \cdot 10\text{^}(pH - pK) \tag{C.5}$$

The root-finding method fsolve in MATLAB was then applied to find the point where these two curves meet, which gives the arterial values for HCO_3^- concentration and pH.

Appendix D

Full general linear mixed effects regression model results for ventilation

	Ventilation		
Fixed effects	*Coefficient*	*t*	*p*
Intercept	3.94	61.89	<0.01
Power	0.20	1.04	0.30
HR	0.38	2.34	0.02
Sex (male)	0.14	1.89	0.06
Age	0.01	0.36	0.72
Lactate	-0.01	-0.20	0.84
H^+	-0.05	-2.54	0.01
HCO_3^-	-0.18	-3.74	<0.01
βHB	-0.03	-1.27	0.21
K^+	0.07	3.59	<0.01
Power x HR	-0.19	-4.68	<0.01
Power x sex (male)	0.28	1.40	0.16
HR x sex (male)	-0.13	-0.76	0.45
Random effects:	*Intercept*	*Residual*	
Subject intercept	0.00	59.49	
Model R^2	0.98		

Appendix E

Full general linear mixed effects regression model results for leg discomfort, anxiety of leg discomfort and anxiety of breathing

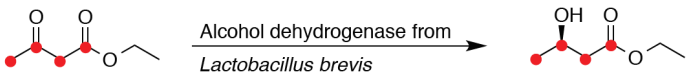
	Breath anxiety			**Leg discomfort**			**Leg anxiety**		
Fixed effects	*Coeff.*	*T*	*p*	*Coeff.*	*T*	*p*	*Coeff.*	*T*	*p*
Intercept	0.48	0.81	0.42	1.39	4.67	<0.01	0.17	0.31	0.76
Power	0.36	0.43	0.67	0.73	1.65	0.10	-0.89	-1.83	0.07
HR	0.22	0.27	0.79	0.48	1.08	0.28	2.13	3.61	<0.01
Sex (male)	-0.03	-0.08	0.94	0.03	0.11	0.92	0.88	1.51	0.13
H^+	0.37	2.90	<0.01	0.14	1.96	0.05	0.30	4.66	<0.01
Lactate	-0.02	-0.19	0.85	0.03	0.49	0.63	0.23	3.41	<0.01
βHB	-0.04	-0.98	0.32	-0.01	-0.40	0.69	-0.04	-1.43	0.15
Glucose	0.08	1.09	0.27	-0.04	-1.41	0.16	-0.05	-2.02	0.04
Power x HR	-0.16	-0.63	0.53	-0.03	-0.28	0.78	-0.24	-1.68	0.09
Power x sex	-0.33	-0.38	0.70	-0.09	-0.18	0.85	1.75	3.27	<0.01
HR x sex	0.30	0.35	0.73	-0.23	-0.46	0.65	-2.01	-3.10	<0.01
Power x H^+	-0.23	-2.06	0.04	-0.15	-2.40	0.02	-0.35	-5.57	<0.01
Random effects:	*Intercept*	*Residual*		*Intercept*	*Residual*		*Intercept*	*Residual*	
Subject intercept	0.18	1.68		0.05	1.10		0.28	0.64	
Model R^2		0.74			0.9			0.81	

Appendix F

Preparation and synthesis of the labeled ketone monoester

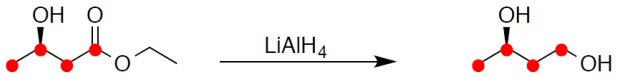
The two tetra-13C-labelled ketone monoester used was synthesised from a total of 47 g of ethyl acetoacetate-1,2,3,4-$^{13}C_4$ purchased form Cambridge Isotope Laboratories, USA.

Synthesis of ethyl (R)-3-hydroxybutyrate-1,2,3,4-$^{13}C_4$: The synthesis of the first component of the ^{13}C-labelled ketone monoester, ethyl (R)-3-hydroxybutyrate-1,2,3,4-$^{13}C_4$, was carried out using the asymmetric reduction catalysed by alcohol dehydrogenase derived from lactobacillus (ADH-LB), from the starting reagent ethyl acetoacetate-1,2,3,4-$^{13}C_4$ (reaction 1).



Reaction 1

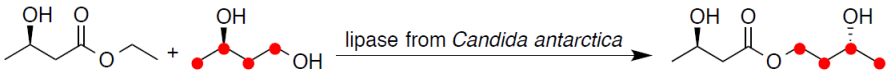
Synthesis of (R)-butane-1,3-diol-1,2,3,4-$^{13}C_4$: Half of the product prepared by the ADH-LB reduction (ethyl ester reaction 1, above) was used to synthesise the second component of the ^{13}C-labelled ketone monoester, (R)-butane-1,3-diol-1,2,3,4-$^{13}C_4$. This reduction was carried out using LiAlH4 as a suspension in anhydrous THF (reaction 2).



Reaction 2

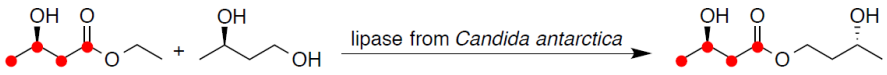
Finally, both *(R)*-butane-1,3-diol-1,2,3,4-$^{13}C_4$, and ethyl *(R)*-3-hydroxybutyrate-1,2,3,4-$^{13}C_4$ from reactions 1 and 2 were transferred to Sterling Pharmaceutical solutions Ltd (UK) for final ketone monoester transesterification under good manufacturing practice grade conditions. Briefly, ketone monoester was prepared from ^{13}C-labelled *(R)*-butane-1,3-diol using an excess of unlabeled ethyl *(R)*-3-hydroxybutyrate (reaction 3). The reaction was carried out at 20–25 °C under reduced pressure (≈2

mbar) for 5 h in order to distil off the ethanol and drive the reaction forward. The product was subsequently purified by short-path distillation in two batches using Kugelrohr apparatus.



Reaction 3

Ketone monoester was prepared from ^{13}C-labelled ethyl *(R)*-3-hydroxybutyrate using an excess of unlabeled *(R)*-butane-1,3-diol (reaction 4). The reaction was carried out at 20–25 °C for 4 h at ≈5 mbar and then for 3.5 h at ≈2 mbar. Again, the product was purified by short-path distillation using Kugelrohr apparatus.



Reaction 4

Equal aliquots of the final products of both reactions 3 and 4 (>98% purity) were incorporated with the native (unlabeled) ketone monoester during drink preparation to achieve a final drink ^{13}C enrichment of approximately 25%.

Appendix G

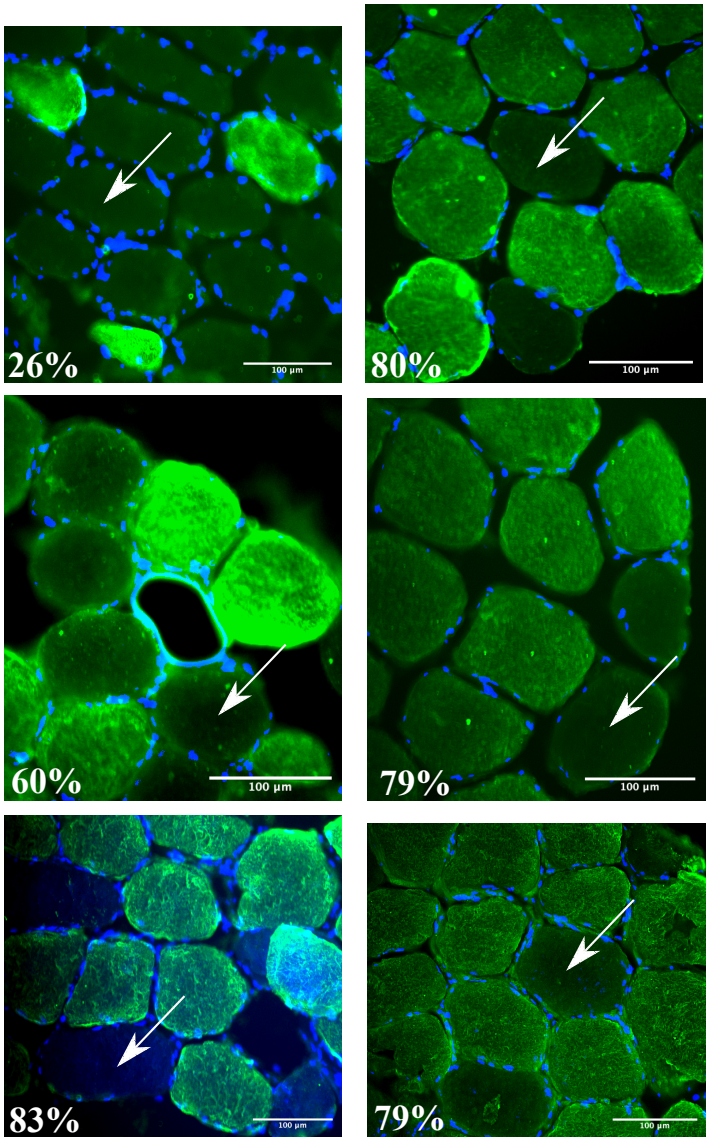
Components of exercise exertion and emotional responses

Exercise measurements are presented as time points with the relative workload in parentheses.

	Measurement	Control	Low ketosis	High ketosis
Leg discomfort (0 - 10)	Pre-drink (rest)			
	Post-drink (rest)			
	15 min (25%)	0.25 ± 0.50	0.75 ± 0.96	0.25 ± 0.50
	20 min (25%)	0.25 ± 0.42	0.50 ± 0.84	0.17 ± 0.41
	35 min (50%)	2.00 ± 1.63	2.13 ± 2.17	2.00 ± 1.41
	40 min (50%)	1.42 ± 1.56	1.83 ± 1.75	1.50 ± 1.22
	55 min (75%)	5.50 ± 1.29	5.25 ± 1.26	4.75 ± 2.63
	60 min (75%)	5.33 ± 1.86	4.42 ± 2.50	4.83 ± 2.93
Leg anxiety (0 - 10)	Pre-drink (rest)			
	Post-drink (rest)			
	15 min (25%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	20 min (25%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	35 min (50%)	0.88 ± 1.03	0.75 ± 1.50	0.75 ± 0.96
	40 min (50%)	0.58 ± 0.92	0.58 ± 1.20	0.50 ± 0.84
	55 min (75%)	2.50 ± 1.91	2.25 ± 2.22	2.63 ± 2.50
	60 min (75%)	2.00 ± 2.53	1.75 ± 2.23	2.33 ± 3.27
Breathlessness (0 - 10)	Pre-drink (rest)			
	Post-drink (rest)			
	15 min (25%)	0.00 ± 0.00	0.50 ± 0.58	0.00 ± 0.00
	20 min (25%)	0.17 ± 0.41	0.33 ± 0.52	0.00 ± 0.00
	35 min (50%)	1.88 ± 0.85	1.50 ± 1.78	1.25 ± 0.50
	40 min (50%)	1.75 ± 0.76	1.25 ± 1.44	1.00 ± 0.63
	55 min (75%)	5.00 ± 0.82	4.75 ± 1.04	5.13 ± 1.03
	60 min (75%)	4.67 ± 1.63	4.50 ± 1.52	4.08 ± 1.91
Breathing Anxiety (0 - 10)	Pre-drink (rest)			
	Post-drink (rest)			
	15 min (25%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	20 min (25%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	35 min (50%)	0.13 ± 0.25	0.50 ± 1.00	0.38 ± 0.48
	40 min (50%)	0.42 ± 0.66	0.50 ± 1.22	0.25 ± 0.42
	55 min (75%)	2.25 ± 0.96	2.00 ± 1.63	2.75 ± 2.36
	60 min (75%)	1.83 ± 1.94	1.75 ± 2.09	2.08 ± 2.42

Appendix H

Example images of muscle fibre staining for each participant



Vastus lateralis biopsy samples stained with an anti-slow skeletal myosin heavy chain primary antibody (fluorescent green). Nuclei are represented in blue (stained with DAPI). Example non-stained fibres (representing fast-glycolytic (IIa) and fast (IIb) fibres) are indicated with the white arrow. The percentage values represent the proportion of slow-oxidative fibres (minimum fibre count of 50).

Appendix I

Daily diet diary for a participant undertaking a ketogenic diet

Item	Mass/Volume	Fat *(kcal)*	CHO *(kcal)*	Protein *(kcal)*	Total *(kcal)*
Coffee with double cream	30mL	137	2	2	141
	1 cup	0	11	0	11
Hard-boiled egg	1	37	0	30	67
Sainsbury's coconut milk	193mL	316	22	7	345
Tesco chia seeds	13g	41	2	10	53
Desiccated coconut	10g	58	3	3	64
Coffee with double cream	30mL	137	2	2	141
	1 cup	0	11	0	11
Koko unsweetened coconut milk	500mL	72	4	4	80
Macadamia nuts	100g	682	21	32	735
Coffee with cream	30mL	137	2	2	141
	1 cup	0	11	0	11
Asparagus	77g	5	6	9	20
Smoked mackerel filets	80g	184	2	72	258
Rocket	60g	3	9	5	17
Closed cup mushrooms	102g	2	1	4	7
Halloumi	76g	168	2	67	237
Hard-boiled egg	2	74	0	60	134
Tea with double cream	30mL	137	2	2	141
Mayonaise	30g	189	19	1	209
Lurpak butter	13g	96	0	0	96
Avocado	1 med	189	48	12	249
Tea with double cream	30mL	137	2	2	141
		2801	**182**	**326**	**3309**

Appendix J

Full blood substrate data

Blood substrates measured at rest (BL), during steady state exercise (70% $VO_{2\ Max}$) and at the maximum effort achieved during an incremental exercise test in the exogenous ketosis, CHO and endogenous ketosis conditions.

		Ex. ket.		**CHO**		**End. ket.**	
		BL	*PI*	*BL*	*PI*	*BL*	*PI*
βHB (mM)	BL	0.07±0.06	0.17±0.12	0.10±0.14	0.07±0.04	0.13±0.18	4.14±1.40
Lactate (mM)	BL	1.30±0.30	1.06±0.28	1.66±0.95	1.04±0.48	0.91±0.24	1.25±0.25
	30	4.83±2.10	3.34±1.60	5.52±1.16	3.37±1.21	5.43±3.85	2.25±0.61
	60	3.87±1.42	3.33±1.44	4.93±1.06	3.10±0.97	3.76±2.73	2.89±1.03
	90	3.69±1.24	3.77±1.73	4.58±0.88	3.15±1.02	NA	NA
	Max	4.73±2.74	6.35±3.27	6.90±1.96	8.22±2.92	NA	NA
Glucose (mM)	BL	6.40±1.26	6.92±1.25	5.91±1.43	5.67±1.18	6.00±1.12	5.61±1.02
	30	7.64±0.98	7.11±0.63	5.89±1.29	6.01±0.63	6.40±1.39	4.79±1.35
	60	7.15±1.07	6.69±0.84	5.70±1.34	5.61±1.00	5.87±0.81	4.05±1.02
	90	5.94±1.15	5.76±1.15	5.12±1.11	5.14±0.81	NA	NA
	Max	4.94±1.32	4.70±1.03	4.22±0.78	4.35±1.02	NA	NA
NEFA (mM)	BL	0.21±0.14	0.32±0.09	0.17±0.13	0.38±0.22	0.16±0.07	0.72±0.33
	30	0.22±0.10	0.36±0.14	0.30±0.31	0.44±0.18	0.14±0.10	0.65±0.36
	60	0.26±0.10	0.40±0.12	0.37±0.22	0.53±0.20	0.23±0.16	0.66±0.23
	90	0.31±0.09	0.41±0.15	0.59±0.29	0.66±0.24	NA	NA
	Max	0.29±0.09	0.32±0.09	0.41±0.21	0.44±0.22	NA	NA
TRIG (mM)	BL	0.56±0.13	0.54±0.14	0.71±0.24	0.83±0.14	0.89±0.25	0.94±0.36
	30	0.78±0.14	0.87±0.17	0.91±0.19	1.06±0.19	0.91±0.29	1.44±0.27
	60	0.92±0.18	0.94±0.22	1.11±0.23	1.21±0.23	0.89±0.28	1.75±0.23
	90	1.03±0.26	1.02±0.23	1.25±0.28	1.23±0.51	NA	NA
	Max	0.88±0.20	0.79±0.11	1.08±0.32	1.13±0.10	NA	NA

Appendix K

Full cardiorespiratory data

Cardiorespiratory measures measured at rest (BL), during steady state exercise (70% $VO_{2\ Max}$) and at the maximum effort achieved during an incremental exercise test in the exogenous ketosis, CHO and endogenous ketosis conditions.

		Ex. Ket.		**CHO**		**End. Ket.**	
		BL	*PI*	*BL*	*PI*	*BL*	*PI*
VO_2 ($L.min^{-1}$)	BL	0.28±0.05	0.31±0.09	0.31±0.04	0.34±0.06	0.30±0.10	0.25±0.04
	30	3.52±0.75	3.28±0.89	3.83±0.38	3.55±0.34	3.29±0.47	3.41±0.56
	60	3.41±0.78	3.22±0.68	3.71±0.61	3.44±0.27	3.32±0.63	3.36±0.62
	90	3.63±0.82	3.45±0.78	3.88±0.60	3.47±0.27	NA	NA
	Max	3.69±1.01	4.11±1.27	4.25±0.82	4.26±0.56	NA	NA
V_E ($L.min^{-1}$)	BL	8.4±1.9	9.2±1.9	9.8±2.2	11.2±3.4	9.3±2.6	8.5±2.2
	30	89.7±14.4	94.7±24.4	99.6±7.2	88.3±6.7	84.1±11.2	85.8±11.9
	60	90.2±19.7	86.9±20.9	98.5±11.8	85.8±9.1	91.2±12.2	92.2±14.4
	90	99.1±23.2	90.0±13.5	105.9±8.7	89.1±6.6	NA	NA
	Max	105.0±34.0	114.4±31.8	131.5±15.9	139.8±19.0	NA	NA
RER	BL	0.81±0.05	0.79±0.08	0.75±0.05	0.76±0.03	0.79±0.07	0.75±0.11
	30	0.92±0.06	0.88±0.05	0.85±0.04	0.83±0.04	0.91±0.04	0.78±0.06
	60	0.90±0.05	0.88±0.03	0.85±0.05	0.84±0.04	0.93±0.05	0.81±0.04
	90	0.91±0.04	0.88±0.06	0.86±0.05	0.85±0.06	NA	NA
	Max	0.92±0.02	1.00±0.09	0.92±0.05	0.96±0.07	NA	NA
HR (BPM)	BL	50.7±5.4	49.3±6.9	54.1±6.8	53.4±4.0	59.5±10.3	59.9±7.4
	30	160.7±8.6	153.4±8.4	155.4±9.3	146.3±6.2	161.0±12.20	161.6±16.1
	60	165.3±6.8	155.7±9.7	161.0±8.8	147.1±5.2	166.3±12.6	168.1±12.4
	90	167.7±7.8	157.8±8.1	160.1±16.8	148.3±7.4	NA	NA
	Max	176.0±12.9	180.0±8.9	177.3±9.7	170.4±9.9	NA	NA

Appendix L

Full glycolytic, TCA cycle and carnitine metabolite data

Intramuscular glycolytic and tricarboxylic acid cycle intermediates. Data presented are log(10) transformed arbitrary units (excluding ratios).

	Exogenous ketosis				**Carbohydrate**				**Endogenous ketosis**			
	BL		**PI**		**BL**		**PI**		**BL**		**PI**	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Fructose 6-phosphate	5.24 ± 0.18	5.36 ± 0.38	5.02 ± 0.19	5.44 ± 0.36	5.24 ± 0.21	5.53 ± 0.15	5.05 ± 0.15	5.61 ± 0.09	4.97 ± 0.22	5.31 ± 0.20	4.87 ± 0.24	4.90 ± 0.25
3-Phosphoglyceric acid	3.93 ± 0.06	4.02 ± 0.05	3.93 ± 0.04	4.10 ± 0.10	3.95 ± 0.05	4.13 ± 0.06	3.94 ± 0.07	4.17 ± 0.07	4.48 ± 0.14	4.39 ± 0.23	4.36 ± 0.20	4.39 ± 0.21
Phosphoenolpyruvic acid	2.99 ± 0.29	2.77 ± 0.46	2.76 ± 0.21	2.85 ± 0.29	2.85 ± 0.17	2.79 ± 0.31	2.88 ± 0.20	2.90 ± 0.23	3.08 ± 0.13	2.95 ± 0.21	2.71 ± 0.40	2.89 ± 0.18
Pyruvic acid	4.33 ± 0.25	4.28 ± 0.46	4.46 ± 0.19	4.31 ± 0.12	4.36 ± 0.13	4.37 ± 0.17	4.52 ± 0.24	4.35 ± 0.13	4.09 ± 0.11	4.18 ± 0.08	4.09 ± 0.11	4.26 ± 0.12
Σ Glycolytic intermed.	***5.32* ± *0.16***	***5.43* ± *0.36***	***5.16* ± *0.14***	***5.51* ± *0.32***	***5.32* ± *0.17***	***5.58* ± *0.13***	***5.20* ± *0.12***	***5.65* ± *0.09***	***5.14* ± *0.17***	***5.39* ± *0.17***	***5.05* ± *0.20***	***5.10* ± *0.17***
Citric acid	4.74 ± 0.06	4.87 ± 0.23	4.72 ± 0.07	4.99 ± 0.13	4.78 ± 0.09	4.90 ± 0.16	4.79 ± 0.06	5.01 ± 0.08	4.90 ± 0.07	5.01 ± 0.15	5.08 ± 0.43	5.07 ± 0.15
2-Oxoglutaric acid	2.63 ± 0.04	2.64 ± 0.09	2.67 ± 0.06	2.63 ± 0.05	2.71 ± 0.03	2.65 ± 0.10	2.73 ± 0.06	2.66 ± 0.04	1.97 ± 0.07	1.92 ± 0.06	1.92 ± 0.06	1.94 ± 0.05
Succinic acid	1.76 ± 0.06	1.77 ± 0.04	1.82 ± 0.04	1.76 ± 0.07	1.86 ± 0.02	2.01 ± 0.41	1.87 ± 0.04	1.83 ± 0.04	3.93 ± 0.13	4.08 ± 0.15	3.84 ± 0.07	4.11 ± 0.20
Maleic acid	3.78 ± 0.09	3.83 ± 0.04	3.70 ± 0.09	3.97 ± 0.11	3.70 ± 0.05	3.95 ± 0.16	3.78 ± 0.09	3.95 ± 0.09	3.00 ± 0.53	2.92 ± 0.27	2.82 ± 0.13	2.85 ± 0.10
Σ TCA cycle intermed.	***4.79* ± *0.05***	***4.91* ± *0.21***	***4.76* ± *0.07***	***5.04* ± *0.12***	***4.82* ± *0.08***	***4.95* ± *0.15***	***4.84* ± *0.06***	***5.05* ± *0.07***	***4.96* ± *0.07***	***5.07* ± *0.14***	***5.11* ± *0.20***	***5.10* ± *0.17***

Intramuscular carnitine derivatives. Data presented are log(10) transformed arbitrary units.

	Exogenous ketosis				**Carbohydrate**				**Endogenous ketosis**			
	BL		**PI**		**BL**		**PI**		**BL**		**PI**	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Acetyl-/free-carnitine	0.60 ± 0.07	0.84 ± 0.24	0.71 ± 0.23	0.87 ± 0.16	0.66 ± 0.20	0.88 ± 0.11	0.56 ± 0.10	0.75 ± 0.11	0.51 ± 0.19	0.87 ± 0.26	0.63 ± 0.34	0.72 ± 0.32
L-Carnitine	2.16 ± 0.02	2.10 ± 0.06	2.12 ± 0.03	2.12 ± 0.03	2.13 ± 0.03	2.09 ± 0.03	2.15 ± 0.02	2.11 ± 0.03	2.11 ± 0.04	2.06 ± 0.02	2.09 ± 0.06	2.00 ± 0.11
L-Palmitoylcarnitine	1.75 ± 0.25	1.79 ± 0.18	1.58 ± 0.17	1.74 ± 0.21	1.91 ± 0.22	1.75 ± 0.17	1.63 ± 0.22	1.79 ± 0.26	1.49 ± 0.28	1.60 ± 0.32	1.76 ± 0.29	1.99 ± 0.12
Propionylcarnitine	0.66 ± 0.10	0.91 ± 0.32	0.74 ± 0.33	1.24 ± 0.31	0.60 ± 0.05	1.51 ± 0.16	0.61 ± 0.04	1.12 ± 0.41	0.58 ± 0.32	1.31 ± 0.53	0.39 ± 0.23	1.25 ± 0.63
Σ medium chain carnitines	0.83 ± 0.06	0.91 ± 0.33	0.76 ± 0.19	1.06 ± 0.18	0.83 ± 0.16	1.13 ± 0.12	0.77 ± 0.19	1.00 ± 0.27	0.65 ± 0.27	0.90 ± 0.39	0.72 ± 0.25	1.11 ± 0.41
Σ short chain carnitines	1.59 ± 0.04	1.71 ± 0.23	1.62 ± 0.24	1.86 ± 0.23	1.59 ± 0.05	2.09 ± 0.08	1.59 ± 0.06	1.87 ± 0.22	1.54 ± 0.19	1.92 ± 0.35	1.72 ± 0.25	2.18 ± 0.42

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