Oxford Centre for Diabetes, Endocrinology, and Metabolism

Radcliffe Department of Medicine

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The Effects of Exogenous Ketosis on Human Metabolism, Physiology, and Endurance Exercise Performance

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A Thesis Submitted for the Degree of
Doctor of Philosophy

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This thesis is dedicated to my Grandad
I know you would have been so proud

Abstract

The (R)-3-hydroxybutyl (R)-3-hydroxybutyrate monoester, or ketone monoester (KME), provides an exogenous path to ketosis, with circulating concentrations of the ketone body β-hydroxybutyrate (βHB) elevated beyond those possible during a ketogenic diet within ~30 min of ingestion. As the consequent state of exogenous ketosis is one decoupled from the carbohydrate restriction obligate for endogenous ketosis and hence permits concurrent high-carbohydrate feeding, this presents a unique physiological phenotype. It has been postulated that this state might enhance metabolic health and endurance exercise performance through ketones acting both as an energetically efficient oxidative substrate and due to their pleiotropic signalling properties. This thesis explored how KME ingestion might influence human metabolism and physiology, both at rest and across differing exercise intensities, as well as endurance exercise performance.

Firstly, *Chapter 3* aimed to establish if ingestion of the KME, in a dose-response manner, elicited multimodal endurance exercise performance benefits in a high-carbohydrate postprandial setting. It was discovered that neither a high nor low dose of the KME influenced 10 km running time trial performance after 1 hr of heavy intensity domain cycling compared to placebo. In circulation, though, glucose and lactate levels were lowered, whilst the degree of saturation of fatty acids was increased, proportional to plasma βHB concentration.

Chapter 4 next sought to determine how exogenous ketosis might impact oxygen utilisation efficiency whilst cycling in a state of low-carbohydrate (i.e. glycogen) availability, achieved through prolonged exercise. Delta economy was found to be worsened, with gross economy unaffected, during ketosis compared to placebo across the second half of a 4 hr moderate intensity protocol, despite RER being elevated. In addition, exogenous carbohydrate oxidation rates were supressed post-KME ingestion, whilst plasma glucose and non-esterified fatty acid (NEFA) concentrations were lowered, ketoacidosis depleted blood bicarbonate stores, and ventilatory workload was elevated. Subsequent performance during a higher intensity time-to-exhaustion test did not, however, differ between conditions, though $\dot{V}CO_2$, heart rate, and plasma lactate concentration were lowered by exogenous ketosis.

Finally, *Chapter 5* endeavoured to characterise how exogenous ketosis may modulate circulating and hepatic metabolism across fed and fasted states in healthy individuals at rest. Postprandial hepatic *de novo* lipogenesis (DNL) was determined to be elevated after KME ingestion compared to placebo. Furthermore, plasma insulin concentrations were increased during exogenous ketosis, both postprandially and fasted, with glucose and NEFA levels concomitantly lowered, whilst high density lipoprotein (HDL)-cholesterol levels were elevated by ketosis in the fasted state only. Additionally, after ingestion of the KME, postprandial plasma βHB C_{max} and βHB-AUC were lesser compared to when the same dose was consumed fasted, with T_{max} not differing between the nutritional states.

In conclusion, whilst substantive metabolic and physiological responses to exogenous ketosis were apparent during endurance exercise, performance was either unaffected or worsened, indicating that its application may not be advisable in the settings explored. Furthermore, findings seen after ingesting the KME at rest, in both a fed and fasted state, provide additional context to its proposed therapeutic applications, with future work exploring the impact of prolonged exogenous ketosis warranted.

Declaration

I declare that this thesis has been written solely by myself and that it has not been submitted, in part or in whole, for any previous degree. Research presented is entirely my own, including the execution of:

Study designs	Study visits
Ethics applications	Data analyses
Participant recruitment	Data reporting

With the exception of assistance and/or data provided by the following:
(members of the University of Oxford, unless otherwise stated)

Chapter 3

- Alexander Wythe (DPAG), who prepared KME and placebo drinks to ensure double-blinding, and independently quantified circulating hormone levels from study samples with raw data provided, with permission, for analysis and inclusion in this thesis.
- Nightingale Health Plc (Helsinki, Finland), who conducted NMR spectroscopy plasma metabolomics analyses.

Chapter 4

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- Dr Christopher Day (Department of Earth Sciences), who quantified by EA-IRMS the $^{13}C/^{12}C$ enrichment of maltodextrin powder used in study drinks.

Chapter 5

- Katelyn Ridgway (Nuffield Department of Primary Care Health Sciences), who undertook TAG extractions and GC/GC-MS analyses to assess plasma VLDL fatty acid composition and palmitate deuterium enrichment for six of ten participants, as well as assisting with plasma insulin ELISAs, as part of her undergraduate medical degree Final Honours Scheme (FHS) project.
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Abbreviations

AcAc	Acetoacetate
ACACA	Acetyl-Coenzyme A Carboxylase
AcAc-CoA	Acetoacetyl-Coenzyme A
Ac-CoA	Acetyl-Coenzyme A
ADP	Adenosine Diphosphate
AMP	Adenosine Monophosphate
AMPK	5'Adenosine Monophosphate-Activated Protein Kinase
ANOVA	Analysis of Variance (Test)
ApoB	Apolipoprotein B
ATP	Adenosine Triphosphate
AUC	Area-Under-the-Curve
BD	Butanediol
BDH	3-Hydroxybutyrate Dehydrogenase
BF	Breathing Frequency
βHB	(R-Enantiomer of) Beta-Hydroxybutyrate
BMI	Body Mass Index
bpm	Beats per Minute (Heart Rate)
BW	Bodyweight (kg)
$^{12}C/^{13}C$	Carbon-12/Carbon-13 Isotope
cAMP	Cyclic Adenosine Monophosphate
CHO	Carbohydrate
Chol	Cholesterol
C_{max}	Peak Concentration
CoA	Coenzyme A
CV	Coefficient of Variation
D_2O	Deuterium Oxide (also as 'Heavy Water', ^2H_2O)
DNL	*De Novo* Lipogenesis
DPAG	Department of Physiology, Anatomy, and Genetics (University of Oxford)
EE	Energy Expenditure
EKS	Exogenous Ketone Supplement
ELISA	Enzyme-Linked Immunosorbent Assay
FA	Fatty Acid
FFAR	Free Fatty Acid Receptor
g	Gram
GC-MS	Gas Chromatography-Mass Spectrometry

GDF15	Growth Differentiation Factor-15
GI	Gastrointestinal
G6P	Glucose-6-Phosphate
GPCR	G-Protein Coupled Receptors
Hb	Haemoglobin
HCAR2	Hydroxycarboxylic Acid Receptor 2
HDL	High Density Lipoprotein
HR	Heart Rate
hr	Hour
HSL	Hormone-Sensitive Lipase
IH/IMTAG	Intra-Hepatic/Intra-Muscular Triacylglycerol
KB	Ketone Body
kg	Kilogram
km	Kilometre
KME	Ketone Monoester; D-β-hydroxybutyrate-R 1,3-butandiol monoester (deltaG®)
L	Litre
La	Lactate
LCHF	Low Carbohydrate, High Fat (also as 'Ketogenic') Diet
LDL	Low Density Lipoprotein
LT1	Lactate Threshold 1
M	Molar (mol/L)
MASLD	Metabolic Dysfunction-Associated Steatotic Liver Disease (previously NAFLD)
Max.	Maximum
MCT	Monocarboxylate Transporter
min	Minute
Min.	Minimum
mol	Mole (amount; 6.022×10^{23})
MRS	Magnetic Resonance Spectroscopy
M/PUFA	Mono/Poly Unsaturated Fatty Acid
NAD	Nicotinamide Adenine Dinucleotide
NAFLD	Non-Alcoholic Fatty Liver Disease
NEFA	Non-Esterified Fatty Acid (also as 'Free Fatty Acid' [FFA])
OCDEM	Oxford Centre for Diabetes, Endocrinology, & Metabolism (University of Oxford)
pCO_2/pO_2	Partial Pressure of CO_2/O_2
PDH	Pyruvate Dehydrogenase
PFK	Phosphofructokinase
P_i	Inorganic Phosphate

PLA	Placebo
PO	Power Output
RER	Respiratory Exchange Ratio
RMR	Resting Metabolic Rate
RPE	Rating of Perceived Exertion
rpm	Revolutions per Minute (Cycling Cadence)
SCD	Stearyl-CoA Desaturase
SCOT	Succinyl-CoA:3-Oxoacid CoA Transferase
SD	Standard Deviation
sec	Second
SEM	Standard Error of the Mean
SFA	Saturated Fatty Acid
SM	Skeletal Muscle
SR	Sarcoplasmic Reticulum
T2D	Type 2 Diabetes (Mellitus)
TAG	Triacylglycerol (also as 'TG')
TCA	Tricarboxylic Acid (Cycle)
T_{max}	Time to Peak Concentration (post KME ingestion)
TT	Time Trial
TTE	Time-to-Exhaustion
TTR	Tracer-to-Tracee Ratio
T2D	Type II Diabetes
U	International Units (plasma insulin concentration)
UCP	Uncoupling Protein
USG	Urine Specific Gravity
$\dot{V}CO_2$	Volume of Carbon Dioxide Produced per Minute
V_E	Minute Ventilation
VLDL	Very Low Density Lipoprotein
$\dot{V}O_2$	Volume of Oxygen Utilised per Minute
$\dot{V}O_{2peak}$	Peak Volume of Oxygen Utilised per Minute
V_T	Tidal Volume
VT1	First Ventilatory Threshold
VT2	Second Ventilatory Threshold
W	Watt (Power)
W_{max}	Maximal Power
WU	Warm Up
°C	Degrees Celsius

Chapter 1 - General Introduction

1.1 Historical Context

It has been clear for decades that diet composition influences human exercise capacity and, more broadly, health[1–4]. As moderate-to-high intensity exercise carries a high energetic demand, the long-held view has been that high carbohydrate provision is required for optimal performance due to its oxygen efficiency and superior ability to drive ATP resynthesis as an oxidative substrate[3,5]. However, in the early 1980s notable attention arose surrounding the use of 'low-carbohydrate, high-fat' (LCHF; <50 g/day carbohydrate), or 'ketogenic', diets[6–8] to potentially increase endurance exercise performance. Previously the preserve of clinical applications, for example treating epilepsy[9] and paediatric malabsorption disorders[10], these diets were introduced into the athletic realm with the rationale of enhancing fat oxidation capacity[6,11] alongside the induction of elevated circulating ketone body (KB) levels, or 'ketosis'.

Interest in the use of LCHF diets for exercise waned over the subsequent years as research failed to deliver a body of evidence supporting their ergogenic potential, with carbohydrate restriction appearing to even be detrimental to exercise performance, especially at higher intensities[12]. Within the last decade however, use of the ketogenic diet in endurance sport has returned to the limelight, due in part to its rise in popularity as a diet carrying purported metabolic health and weight management benefits[13,14]. In addition, the focus on chronic carbohydrate restriction, both in research and applied sport, has shifted to performance during extreme duration events (Ironman triathlons, ultramarathons, etc)[15–17], the influence of the duration of dietary adherence on its efficacy[6], and perhaps most of all the role played by KBs specifically[18].

Exogenous ketone supplements (EKS), where KBs or ketone-precursors are ingested rather than production being induced endogenously, have been studied since the 1970s[19] but gained renewed

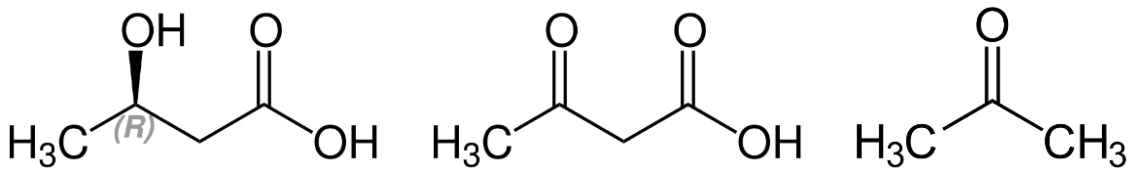
attention in the early 2010s with the development of ketone salts and esters[20,21]. EKS consumers can achieve a ketotic state whilst bypassing the potentially detrimental impacts on metabolism (e.g. dyslipidaemia[22]), and strict lifestyle requirements, of carbohydrate restriction necessary for endogenous ketosis. This novel state of 'exogenous ketosis' carries both major commercial and research implications as it allows for interrogation and application of the metabolic effects of KBs in isolation, in both exercise[23,24] and health[25–29]. Reflecting this, the EKS global market is estimated to reach USD$ 918.1 million by 2030[30], whilst academic research output in the field has sharpy risen in recent years[31–37] .

1.2 Ketone Body Metabolism

1.2.1 Ketone Bodies

KBs are short-chain, water soluble, hydrocarbons named for their ketone moieties and produced hepatically[38] in humans. Ketogenesis is an evolutionarily adaptive survival response, conserved in higher order organisms, where KBs are endogenously synthesised from lipids to act as glucose-surrogate provision for the brain during energetic crisises[1,39]. Ketones have therefore been classed, by some, as the 'fourth macronutrient'[40].

The KBs of biological relevance are β-hydroxybutyrate (βHB), acetoacetate (AcAc), and acetone[38] (*Figure 1.1*), which sit along a spectrum of oxidation states. The R-enantiomer of βHB is the most chemically reduced and makes up ~80% of circulating KBs in humans[23,38], so is considered to have the most relevance to metabolism (βHB henceforth refers to R-βHB unless otherwise stated, see 1.2.7).



β-hydroxybutyrate Acetoacetate Acetone

***Figure 1.1* - Chemical structure of biologically relevant ketone bodies.**
βHB's hydroxyl group can be easily converted into a ketone group, so it is considered a ketone body despite technically lacking a ketone moiety.

Ketones can be viewed teleologically as signals of carbohydrate paucity, thus are closely involved in the integrative metabolic response that functions to spare this scarce and valuable resource during starvation[41]. Accordingly, they act as more than simple oxidisable fuel sources, carrying pleiotropic signalling properties that can alter the metabolism of other substrates, influence whole body metabolic homeostasis, and supress processes such as inflammation via decreasing NLRP3 inflammasome activity[42,43], oxidative stress via inhibiting histone deacetylases (HDACs)[44], and protein catabolism (1.3.4), whilst modulating gene expression in multiple organs[45–48].

1.2.2 Endogenous Ketosis & Ketogenesis

Endogenous ketosis is the hepatic synthesis of KBs, stimulated in healthy individuals by a chronically lowered insulin-to-glucagon ratio. This is sensed by hormone-sensitive lipase (HSL) in adipocytes, and acetyl-CoA (Ac-CoA) carboxylase in hepatocytes, whilst depleted hepatic glycogen stores are detected by HMGCS2[24,38,49–51]. Ketosis is therefore induced during prolonged fasting, carbohydrate restriction, fasted exercise, and pathologically in diabetic ketoacidosis (DKA), see *Table 1.1*. The primary substrates for ketogenesis are fatty acids (FA) with a ~5% contribution from ketogenic amino acids[52]. Thus it has been traditionally viewed as β-oxidation 'spillover'[29] wherein excess Ac-CoA enters the ketogenic pathway to produce βHB and AcAc (*Figure 1.2*), synthesising up to 150 g/day[21,39].

A physiological state of ketosis is established when KB production exceeds breakdown and circulating levels rise. Traditionally defined as blood βHB ≥0.2 mM, more recent proposals employ a 0.5 mM threshold[29,47,48]. KBs act to supress adipose lipolysis (1.3.4), lowering circulating FA availability as a ketogenic substrate, and therefore establishing an inhibitory end-product feedback loop on their own production. This prevents runaway ketogenesis and limits ketosis to ~7.5 mM (outside of DKA, a pathological complication of diabetes mellitus and thus distinct from other ketotic states)[53].

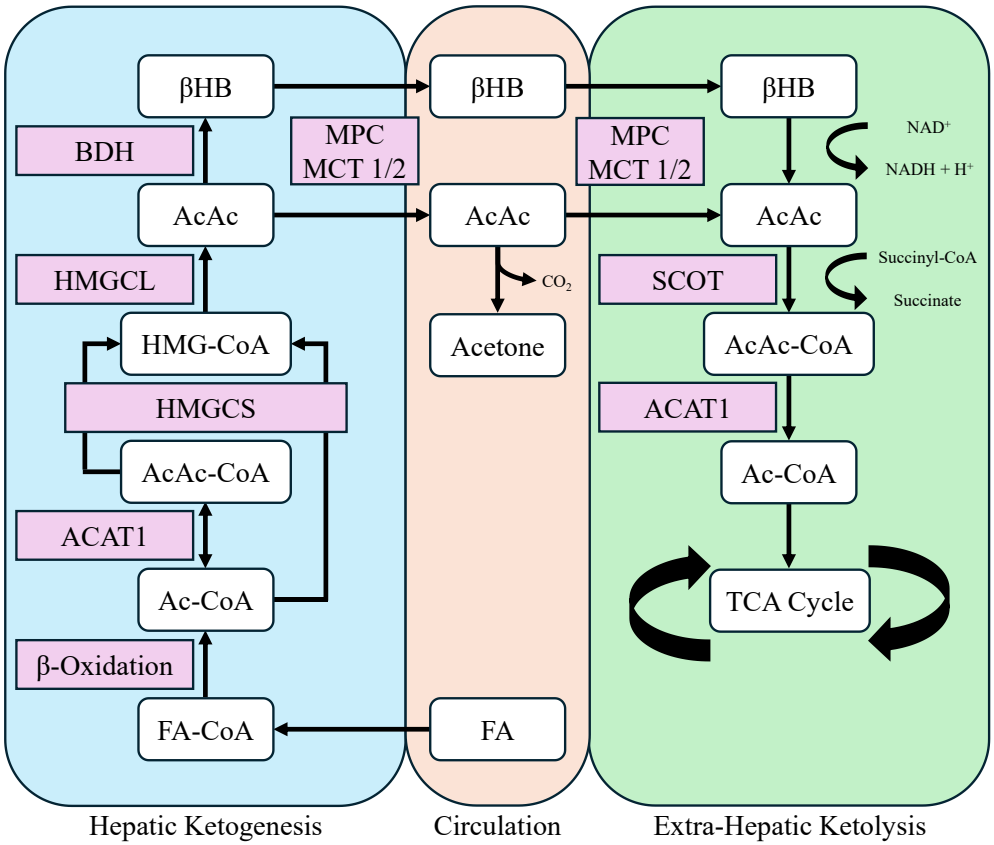


***Figure 1.2* - Overview of ketone body metabolism.**

Hepatic Ketogenesis: Elevated circulating free FAs are taken up from circulation by the liver then converted to fatty acyl CoA (FA-CoA)[52]. Fa-CoA enters mitochondria via CPT1-mediated transport and undergoes β-oxidation to form acetyl-CoA (Ac-CoA). Ketogenesis is stimulated by Ac-CoA synthesis exceeding citrate synthase activity and/or oxaloacetate availability (which is reduced when glucose availability falls as it is utilised for gluconeogenesis) and thus exceeding capacity for incorporation into the tricarboxylic acid (TCA) cycle[38,49,54]. A series of condensation reactions starting with Ac-CoA result in the formation of acetoacetyl-CoA (AcAc-CoA), catalysed by Ac-CoA acetyltransferase 1 (ACAT1). Hydroxymethylglutaryl-CoA (HMG-CoA) is formed from Ac-CoA and AcAc-CoA by hydroxymethylglutaryl-CoA synthase 2 (HMGCS2), at this point irreversibly committing substrate to ketogenesis. HMG-CoA decomposes, cleaved by HMG-CoA lyase (HMGCL), into AcAc and Ac-CoA. Some AcAc enters circulation, but as the NAD^+/NADH coupled equilibrium favours βHB formation, the majority is reduced to βHB, catalysed by 3-hydroxybutyrate dehydrogenase (BDH). βHB is found at a 3-5:1 ratio with AcAc, dependant on the NAD^+/NADH ratio in hepatic mitochondria (larger ratio with greater severity/duration of starvation/carbohydrate restriction)[38]. Circulating Ketosis: βHB and AcAc enter/exit the mitochondria via mitochondrial pyruvate carriers (MPC) and/or the SLC16A family of monocarboxylate transporters (MCT), namely MCT 1 & 2 (potentially also MCT 7). They enter/exit circulation via MCT 1/2[55]. Extra-Hepatic Ketolysis: βHB's obligate metabolic pathway is re-oxidation into AcAc within mitochondria in a NAD^+/NADH near-equilibrium reaction. AcAc is bound to CoA by succinyl-CoA:3-oxoacid CoA transferase (SCOT) with succinyl-CoA acting as a CoA donor, forming AcAc-CoA, this is the rate-limiting step of ketolysis. AcAc-CoA is thiolytically cleaved by Ac-CoA acetyltransferase (ACAT), with the resultant two molecules of Ac-CoA joining the TCA cycle[24]. Non-Oxidative Fates: AcAc may not be oxidised and instead re-enter circulation, where it can be used as substrate for de novo lipogenesis (DNL) and cholesterogenesis[56,57], whilst both βHB and AcAc can also be excreted in urine[29,38]. Acetone is formed from the spontaneous decarboxylation of AcAc and is generally considered to be sequestered into adipose tissue or lost in breath[38], though there is limited evidence that can be utilised as a gluconeogenic substrate[58]. Exercise Training: MCT, BDH, SCOT, and ACAT enzymes have elevated content and activity in endurance exercise trained SM (see 1.2.4).

***Table 1.1** - Approximate ranges of human blood ketone body concentrations found in differing endogenous ketogenic states.*

Metabolic State	Blood Ketone Body Levels (mM)
Postprandial (carbohydrate unrestricted)	<0.1
Overnight fast	0.2 - 0.3
24 hr fast	~1
Post-prolonged exercise	0.3 - 2
Multi-day fast	2 - 3
Multi-week ketogenic diet	3 - 5
1 week fast	5 - 6
>4 week fast	6 - 7.5
Diabetic ketoacidosis	<25

Values are combined concentrations of βHB and AcAc measured in whole blood. Data adapted from: Sherwood et al. 1970[1]; Robinson & Williamson 1980[38]; Evans et al. 2017[24].

1.2.3 Ketone Body Oxidation

The predominant fate of KBs is mitochondrial oxidation, or 'ketolysis'. This occurs in all tissue except for the liver, as hepatocytes carry a very low abundance of SCOT, preventing futile ketogenic-ketolytic cycling[38]. The ketolytic pathway is described in *Figure 1.2*, with the overall reaction for βHB oxidation as follows:

$$\beta HB + NAD^+ + CoA + \text{succinyl-CoA} \rightarrow NADH + H^+ + 2\ \text{Ac-CoA} + \text{succinate}$$

1.2.4 Utilisation in Muscle

A likely prerequisite for KBs being able to influence, potentially beneficially, exercise metabolism is their uptake and utilisation in working muscle. Cardiac tissue is considered the muscle type best able to oxidise ketones[38,59,60], with KBs observed to account for 83% of CO_2 production in a canine heart perfusion model[61]. In contrast, ketolytic enzyme activity is approximately an order of magnitude lower in skeletal muscle (SM) than in the heart[62,63] (BDH: 2.5 vs 0.6 μM/min, SCOT: 26 vs 0.8 μM/min; heart vs SM[38]). However, SM accounts for ~40% of total body mass in non-obese induviduals[64] and therefore represents the largest potential site by mass for ketone uptake and oxidation[65]. Additionally, whilst SM energetic demands are low at rest[49,53], during exercise ATP resynthesis rates can rise over 100-fold[66,67],

utilising >80% of cardiac output (<20% at rest)[68]. Thus, exercise presents a setting where this tissue might carry substantial ketolytic capacity.

Direct *in vivo* investigation of ketone oxidation capacity is however limited, as ketolysis confounds calculation of substrate oxidation rates from whole-body gas-exchange data[69]. Therefore βHB oxidation in humans has only been directly quantified, through use a ^{13}C-βHB tracer, in two exercise studies[70,71]. These reported a ~30-fold increase in βHB oxidation from rest to 75% W_{max} cycling exercise in fasted participants (~0.01 to ~0.30 g/min) contributing ~8.4% to total energy expenditure (EE)[70].

Indirect evidence of KB utilisation by active SM comes via assessment of clearance rates. Balasse & Fery[53] documented a ~40% greater KB clearance into SM upon commencement of exercise in overnight fasted participants. They noted that this pattern was similar to those seen for carbohydrate and fat clearance, thus might be indicative of KBs acting as an oxidative fuel. In the same study however, the capacity of exercise to stimulate uptake lessened as blood ketone levels rose (manipulated by varying fasting duration) and was lost entirely at ~5.5 mM, indicating uptake-saturation occurs at relatively low levels of ketosis[53]. Saturation has subsequently been posited to occur in non-athletes at ~1-2 mM[24] based on this data and that from step-wise βHB infusions[72].

Ketolytic capacity appears to vary between SM fibre types. Activity of key enzymes BDH, SCOT, and ACAT has been shown to be highest in type-I muscle fibres, intermediate in type-IIA, and lowest in type-IIB in rats[63,73], whilst MCT1 protein expression, which correlates with overall oxidative capacity at a tissue-level[74], is highest in type-I fibres. Furthermore, in an aerobic exercise-training rodent model, activity of BDH, SCOT and ACAT was greater in trained, compared to sedentary, SM[73,75,76], with BDH activity found to have increased 3x in type-I and 6x in type-IIA fibres specifically[73]. Moreover, in a perfused hindlimb model the capacity for uptake of KBs at 1 mM total KBs was 33% (total KB), 27% (AcAc), and 53% (βHB) higher in trained compared to untrained rats[77]. These training adaptations could be indicative of KB oxidation representing a limiting factor to metabolic responses to exercise stimuli, functioning to reduce the magnitude of these perturbations to homeostasis during subsequent exercise challenges by elevating scope for ketone utilisation[78].

It is therefore likely that well-trained[79] and type-I fibre-type dominant (i.e. endurance-type)[80–83] athletes have the greatest potential ketolytic capacity, especially during exercise. This has been preliminarily

supported by Dearlove et al.[71] observing a positive relationship ($R^2 = 0.696$) between vastus lateralis biopsy % type-I fibre and maximal βHB oxidation rate.

1.2.5 Utilisation in the Brain

The brain does not oxidise FAs, posited to be an evolutionary adaption to bypass β-oxidation's relatively slow ATP resynthesis rates and associated risk of oxidative stress[84]. When glucose is limited, the brain therefore utilises KBs as a surrogate, facilitating indirect utilisation of fat stores for neural metabolism, with KBs representing >50% of neural O_2 consumption after 5-6 weeks of fasting[85]. This large ketolytic capacity suggests KBs could support maintenance of cognitive capabilities during energetic crises, for example under exercise-induced hypoglycaemia[86], and thus might play a role in decision making, central fatigue[87], and motivation during prolonged or exhaustive exercise[88,89].

1.2.6 Oxidative Advantage of βHB as a Substrate

βHB has been previously posited by some to be the most 'efficient' oxidative substrate, due to a greater energy yield of oxidation than glucose or pyruvate coupled with superior oxygen efficiency compared to FAs[21,59,90–92].

Oxidation of βHB entails a high energy liberation, or 'heat of combustion'[90], due to its highly reduced chemical state. The electrochemical gradient that drives proton flow across the inner mitochondrial membrane, re-synthesising ATP from ADP and inorganic phosphate (P_i), is generated by the differences in potentials of two redox pairs, the NAD^+/NADH and Co-enzyme Q couples. βHB oxidation reduces (lowers) the NAD^+/NADH ratio whilst oxidising the Co-enzyme Q couple. This increases the redox span, therefore potential energy, between sites I and II of the electron transport chain to a greater extent than glucose, pyruvate, or lactate when expressed relative to carbon pairs (*Table 1.2*)[26,90,92]. However, given most of the pyruvate oxidised in muscle is consequent of glycolysis[93], directly comparing βHB and pyruvate oxidation is potentially misleading, with the energy yield disparity much narrower between glucose and βHB. FA oxidation is also notably (~22%) more energy dense[21].

***Table 1.2* - Energy yield of substrate combustion (ΔH°).**

	ΔH° (kcal/mol)	**ΔH° (kcal/mol C_2)**
Glucose	-669.9	-223.6
Pyruvate	-278.5	-185.7
βHB	-487.2	-243.6
Palmitate	-2384.8	-298.0

Data adapted from: Veech 2004[90], Cox & Clarke 2014[21].

An alternative metric for assessing substrate oxidative efficiency is the ratio of ATP produced to oxygen used during oxidative phosphorylation, commonly quantified as the phosphate/oxygen ratio (P/O). P/O represents the number of phosphates that are fixed into ATP for every two electrons that pass through an electron transport chain and are terminated by the reduction of an oxygen atom from molecular O_2[91,94]. This carries elevated relevance during exercise as oxygen delivery to active muscle can be a limiting factor to high intensity performance[95]. βHB and glucose hold similar P/O values whereas FAs, despite being more chemically reduced, carry inferior ratios (*Table 1.3*). This is due to half of the reducing equivalents produced in β-oxidation being in the form of $FADH_2$ rather than NADH, alongside greater intracellular FA concentrations being associated with increased expression of uncoupling proteins (UCP) which dissipate the inner mitochondrial electrochemical gradient[21,31,90]. In addition, the maximal rate of ATP resynthesis from the oxidation of fat is 2-3 times slower than of carbohydrate (0.24 vs 0.51-0.68 mmol/sec/kg)[84,96]. As ATP stores in SM are limited (~5 mmol/kg wet-weight)[97], as exercise intensity, thus ATP resynthesis rates and O_2 demand, increase, FA oxidation is progressively supressed with carbohydrate utilisation predominating[96]. Given βHB's superior P/O relative to FA, ketolysis might act similarly to carbohydrate oxidation, increasingly contributing to EE as exercise work rates rise.

***Table 1.3* - ATP produced per molecule of oxygen used (P/O) during substrate oxidation.**

	P/O (ATP/[1/2 O_2])
Glucose	2.58
βHB	2.50
Fatty Acids	~2.33

Data adapted from: Karwi & Lopaschuk 2022[91], Cotter et al. 2013[98].

An often cited functional example of the oxidative advantage of KBs comes from Sato et al.[59]. In a perfused working rat heart model, addition of KBs to the substrate mix suppressed glycolytic flux but increased hydraulic efficiency (as J/mol of O_2) by 28%. Were KB oxidation to have an analogous impact on SM efficiency in exercise, it might be expected that power output would increase for the same O_2 consumption rate ($\dot{V}O_2$), or $\dot{V}O_2$ be lowered for the same power, i.e. exercise economy[99] would be improved. It is debated as to how applicable this finding is to *in vivo* substrate competition however, with mixed results in healthy and failing human hearts finding that KBs contribute to energy provision and increase cardiac output, but don't affect efficiency[100,101]. These findings also don't appear, within limited existing data, to translate to non-cardiac tissue, with maximal provision of KB oxidation to whole body EE during exercise being <10%[31,70,71], in contrast to the much greater contributions seen in the heart[38]. The implications of this for exercise are explored further in 1.4.3.

1.2.7 R-βHB & S-βHB

βHB exists as two optical enantiomers, R (or D) and S (or L). Both are found naturally in endogenous ketosis, but as S-βHB is formed transiently as a β-oxidation intermediate and constitutes only ~3% of total KBs[102] its metabolism is poorly described. S-βHB likely plays a role in hepatic synthesis of FAs and sterols, but makes little direct contribution to energy production as it is not a substrate for BDH so cannot be oxidised[103], with a large proportion being converted to R-βHB[104]. When S-βHB is ingested exogenously (1.3.1), levels remain elevated for longer than R-βHB, reflecting this lack of oxidative-disposal[23]. It is though able to bind to GPCRs and provoke similar intracellular signalling cascades to R-βHB[105]. S-βHB is not detected by Point-of-Care βHB meters as these use BDH-based amperometric strips to establish whole blood R-βHB, instead requiring analysis via GC-MS with a chiral column[23,106], thus levels are often under-/un-reported.

1.3 Exogenous Ketosis

Consuming EKSs allows for ketosis independent of the potentially confounding dietary manipulations (starvation/LCHF) necessary for endogenous ketosis, with concomitant depletion of glycogen stores and chronically low insulin:glucagon[24]. In contrast, under a state of exogenous ketosis, circulating KBs can be high alongside replete glycogen stores and elevated insulin[107], establishing a unique metabolic phenotype. It also confers enhanced metabolic flexibility compared to endogenous ketosis with greater onset immediacy (minutes *vs* days/weeks), plus superior control over the degree of ketosis attained and the duration for which it is sustained, modifiable by the amount and timing of EKS consumed[23,31,107]. Through decoupling ketosis from a obligate carbohydrate-restricted state, and with the potential for KBs to be oxidised in active muscle, interest for exogenous ketosis in endurance sport has risen dramatically in recent years[31,108].

1.3.1 Types of Exogenous Ketone Supplement

There are three main forms of EKS: ketone salts, ketone esters, and ketogenic precursors. Their composition and efficacy at inducing ketosis are detailed in *Table 1.4*.

***Table 1.4** - Exogenous ketone supplements (EKS) in healthy individuals.*

EKS Type	Composition	Metabolism	R-βHB Achievable (mM)	Onset of Ketosis (min)	Return to Basal [βHB] (hr)	Effects on Endurance Exercise Performance
Ketone Ester						
Ketone Monoester (KME) [20,23,107,109,110]	R-β-hydroxybutyrate R-1,3-butandiol monoester	*Figure 1.3*	6.5	10	~4	1.4.1
Acetoacetate Diester [111]	1,3-butanediol acetoacetate diester	Ester bond cleaved in gut AcAc and 1,3-BD taken up & metabolised as in *Figure 1.3*	0.3 - 0.6	30	~3.5	One study[111]: negative impact, confounded by severe GI issues
C6 Ketone Diester [27,106,112,113]	Bis hexanoyl R-1,3-butanediol	Ester bond cleaved in gut Hexanoic acid metabolised as a MCFA 1,3-BD taken up & metabolised as in *Figure 1.3*	1.7	30	~4	No applicable studies
Ketone Salts						
Ketone Salt [31,114,115]	$Na^+/K^+/Ca^{2+}$ & R/S-βHB	Uptake as free βHB into circulation via MCTs High salt load, therefore dangerous if preexisting kidney issues	0.4 - 1	20	2 - 2.5	Impaired or no effect, high incidence of GI issues[31] Largely racemic mixes (50:50 S-βHB to R-βHB; 1.2.7 R-βHB & S-βHB)
Ketogenic Precursors						
Butanediol (BD) [116–118]	R/S-1,3-butanediol *or* R-1,3-butanediol	Converted to β-hydroxybutyraldehyde in the liver, oxidised to R,S-βHB via alcohol and aldehyde dehydrogenases as in *Figure 1.3*	0.8 - 1.4	30	>3.5	Two studies: no effect on cycling TT performance[116,118] GI issues & narcotic effects at high doses, 'euphoria' reported in one study[118]
Medium chain fatty acids (MCFA) Medium chain triglycerides (MCT) [31,119,120]	6-12 carbon chain fatty acids/ triglycerides	Hepatically metabolised to acetyl CoA then to KBs, as FAs are metabolised in *Figure 1.2*	0.5 - 1.5	30	>2	Equivocal: generally low quality evidence, high incidence of GI issues[31]

'R-βHB Achievable' reflects the largest concentration seen in existing literature using an EKS dose tolerable for exercise. 'Onset of Ketosis' & 'Return to Basal' assume 350 mg/kg·BW (or closest applicable) dose ingested in a fasted and rested state in healthy individuals. GI, gastrointestinal; TT, time trial.

1.3.2 The Ketone Monoester

The R-3-hydroxybutyl R-3-hydroxybutyrate monoester, or ketone monoester (KME; *Figure 1.4*), is comprised of R-βHB and R-1,3-butanediol (1,3-BD) molecules bound by an ester bond[20] and is commercially available as '*deltaG*'. It has a molecular weight of 176, is a viscous liquid at room temperature (1.0731 g/mL), and carries an 'extreme bitter' taste[107]. An overview of its metabolism after ingestion can be found in *Figure 1.3*.

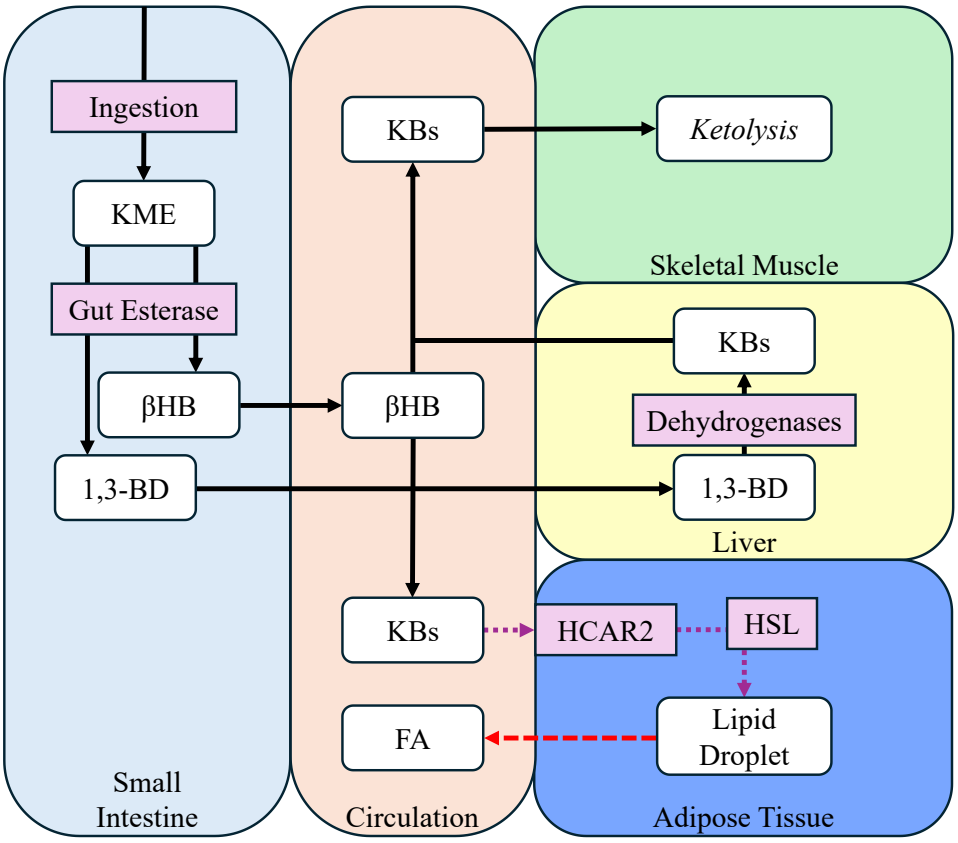


***Figure 1.3* - Overview of ketone monoester metabolism.**

Purple line with short dashes, inhibitory action.
Red line with long dashes, downregulated pathway.

After the ketone monoester (KME) enters the small intestine, the ester bond is hydrolysed by non-specific gut carboxylesterases, leaving βHB & R-1,3-butanediol (1,3-BD) which both enter circulation via MCTs. 1,3-BD undergoes first pass hepatic stepwise oxidation into 3-hydroxybutanal[110] via alcohol dehydrogenase, the rate limiting step[121], and then into βHB and AcAc via aldehyde dehydrogenases and BDH[110,122–124]. In circulation, the βHB:AcAc ranges between ~6:1 when ingested at rest & fasted, ~4:1 when at rest & fed[23], & ~2:1 during exercise when fed[125]. βHB & AcAc can enter extra-hepatic tissue, e.g. skeletal muscle, from circulation for ketolysis & oxidation (1.2.3). They can additionally act antilipolytically on adipocytes by antagonistically binding to hydroxycarboxylic acid receptor 2 (HCAR2) receptors which reduces hormone-sensitive lipase (HSL) activity[105] (1.3.4), reducing circulating free FA levels, and thus acting to supress endogenous ketone body production.

The KME is the only EKS used for research presented in this work, therefore the remainder of this thesis will focus on KME use, and accordingly exogenous ketosis/ketones refers to that/those delivered by the KME unless otherwise stated. Furthermore, as KME ingestion results in >99% of circulating βHB being found as R-βHB[20,109,110], βHB refers to R-βHB in this thesis unless otherwise stated.

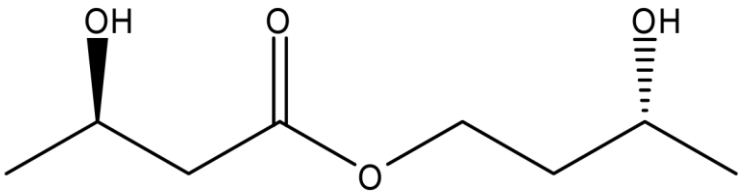


***Figure 1.4* - Chemical structure of the R-3-hydroxybutyl R-3-hydroxybutyrate ketone monoester.**

1.3.3 Influence on Carbohydrate Metabolism

Exogenous ketosis markedly influences carbohydrate metabolism by suppressing glycolysis and lowering circulating glucose levels. Adaptively these metabolic shifts act to spare finite carbohydrate stores during starvation[1,39].

Glycolysis & Glycogenolysis

Observations of ketones inhibiting glycolysis within the heart[59,60,126,127], SM[107,128], and brain[129,130] have been made since the 1950s[131]. As detailed in *Figure 1.5*, KBs are proposed to supress carbohydrate utilisation through inhibition of pyruvate dehydrogenase (PDH) and/or phosphofructokinase (PFK), with lipid (and potentially ketone) oxidation concomitantly increasing to meet energetic demands[21,65,107]. This might reflect ketones' evolutionary origin in signalling an energetic crisis during starvation, where the limited remaining carbohydrate is preserved[38,45]. Thus, KB oxidation may rebalance the Randle ('glucose-FA') cycle[132], a model of substrate level inhibition exhibiting hierarchical preference for oxidative phosphorylation of FAs over carbohydrates, to even more greatly favour β-oxidation. This glycolytic inhibition is suggested to be recapitulated even during exercise, where carbohydrate gains dominance proportional to intensity[96,107,133,134].

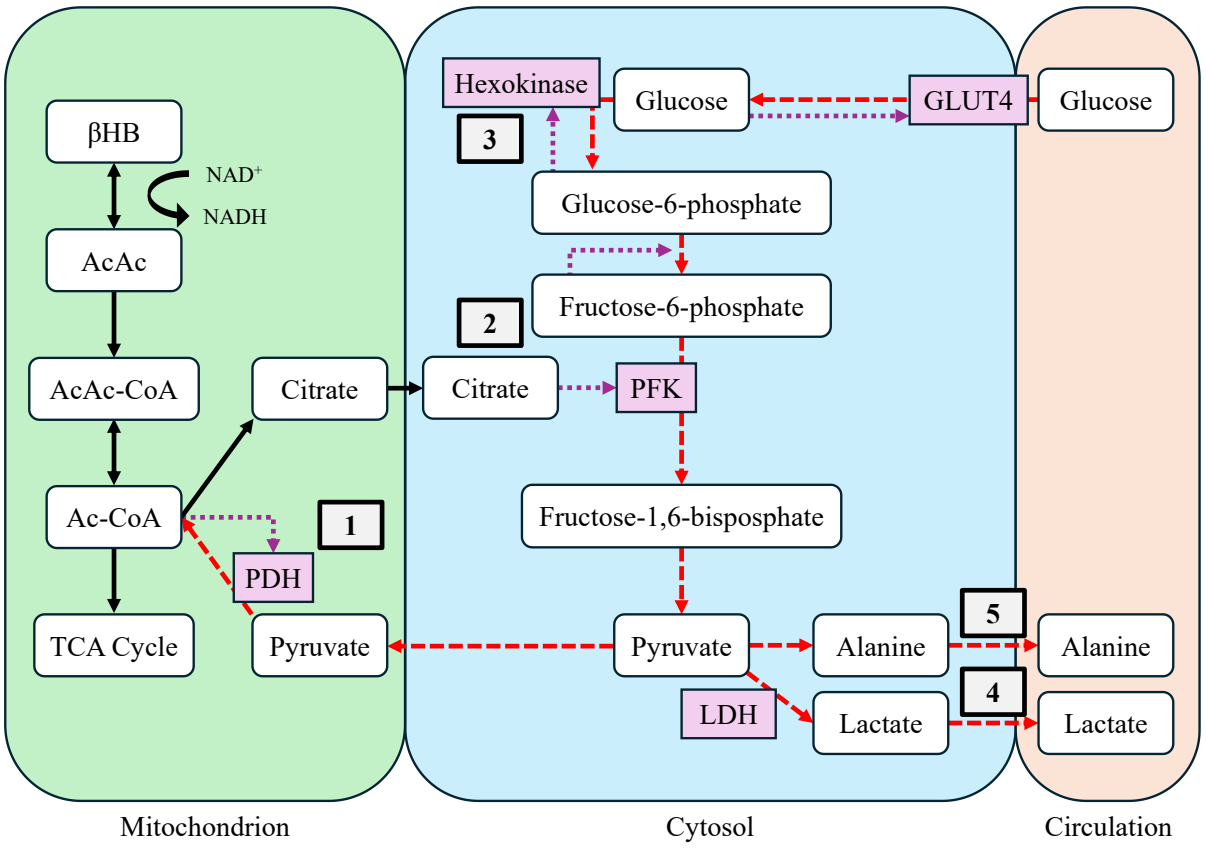


Figure 1.5 *- **Mechanisms by which ketone oxidation might inhibit glycolysis.***

Purple line with short dashes, inhibition of an enzyme or pathway.

Red line with long dashes, downregulated pathway.

Ketolysis can act to inhibit glycolysis by acting on a number of metabolic pathways:

1. Mitochondrial acetyl-CoA (Ac-CoA) accumulation from ketone oxidation results in elevated Ac-CoA/CoA and NADH/NAD+ ratios, which supress pyruvate dehydrogenase (PDH) activity, bottlenecking pyruvate formation from glycolysis (from plasma glucose and/or glycogenolysis)[21]. This inhibitory process mirrors that described in the Randle cycle[127], in which it is FA (rather than ketone) oxidation which drives Ac-CoA accumulation and thus confers substrate-hierarchy preference for oxidation of FAs when availability is high.

2. Excess Ac-CoA (that which is not incorporated into the TCA cycle) is converted to citrate[60,73], which may inhibit cytosolic phosphofructokinase (PFK) activity.

3. Consequential upstream accumulation of glucose-6-phosphate inhibits hexokinase activity, causing glucose to accumulate and therefore reduces entry into the cell from circulation through GLUT4 transporters[21,135].

4. Reductions in glycolysis and/or glycogenolysis decrease lactate dehydrogenase (LDH) flux, lowering intra-/extra-cellular lactate levels[107].

5. Diminished availability of pyruvate for transamination with amino acids (e.g. glutamate) reduces alanine formation, lowering circulating availability of this gluconeogenic substrate[136].

In an exercise setting, evidence for the ability of KBs to 'spare'[137] SM glycogen via inhibition of glycolysis (thus glycogenolysis), and inversely elevate FA oxidation, is mixed. Seminal work by Cox et al.[107] evidenced this proposed re-tooling of the Randle cycle with findings of reduced intra-muscular

glycolytic intermediates and increased acyl-carnitine species, alongside histological indications of reduced glycogen usage and concomitant elevated intramuscular triacylglycerol (IMTAG) usage, after KME and carbohydrate (KME+CHO) ingestion compared to a carbohydrate-only (CHO-only) placebo control across 2 hr of 70% $\dot{V}O_{2max}$ cycling. As muscle glycogen is widely accepted as a potential limiting factor for heavy and moderate intensity domain exercise[138–143], the ability to spare its usage was posited to represent a metabolic, and thus performance, advantage of exogenous ketosis. However, less carbohydrate was provisioned under KME+CHO than CHO-only (conditions were isocaloric) which might in-part explain the reduction in glycolytic intermediates[144,145]. Participants also commenced exercise fasted and in-exercise carbohydrate was sub-optimal[3], therefore direct applicability of these findings to real-world (i.e. postprandial and carbohydrate-optimised) exercise may be limited.

A follow up study where participants undertook 3 hr of 'intermittent'[146] cycling followed by a 15 min time trial (TT), proceeding an ecologically valid high-carbohydrate breakfast and with in-exercise carbohydrate provision matched between conditions, saw a trend ($p = 0.08$) for greater SM glycogen depletion under the KME+CHO condition compared to CHO-only, with IMTAG unaffected[146]. This contrasting outcome suggests that the capacity for KBs to alter substrate-hierarchy might be lost, or even reversed, when carbohydrate availability is high (1.4.2). The authors[146] also questioned the validity of the semi-quantitative PAS stains[147] used by Cox et al.[107] which lack specificity for glycogen[148], asserting that the dry-weight enzymatic fluorometric assay they themselves employed carries greater validity. This dry-weight assessment itself, however, also risks diminished accuracy compared to wet-weight analysis due to body water variability[149]. Accordingly, Howard et al.[150] instead utilised a dual-tracer approach of a primed continuous ^{2}H-glucose infusion, alongside exogenous ^{13}C-glucose, across a 90 min ~54% $\dot{V}O_{2peak}$ treadmill weighted-run, commenced fasted with 30 g/hr carbohydrate provision. They observed that neither exogenous or circulating glucose oxidation, nor metabolic clearance rate (a marker of glucose uptake), differed between KME+CHO and CHO conditions. Reflecting this, plasma metabolomic data did not reveal any substantial shifts in carbohydrate metabolism, whilst subsequent time-to-exhaustion (TTE) and TT performance was impaired under KME+CHO, not enhanced as in Cox et al.[107] (*Table 1.5*). Despite participants undertaking testing in similar feeding states, these findings therefore sit in direct disagreement with Cox et al.[107] at both performance and metabolic levels. Exercise

modalities were different, however, and relative exercise intensity was lower in Howard et al.[150] with consequent lesser rates of carbohydrate utilisation[96] potentially leaving diminished scope for influence by ketosis.

A number of investigations have used circulating lactate as an indirect marker of inhibited glycolysis during exercise, as it is regularly shown to be lowered under exogenous ketosis[24,31,71,107,151,152] (*Figure 1.5, Point 4*). This observation is not universal however, especially at moderate intensities[146,153,154]. Additionally, saturation of MCTs by KBs reduces lactate efflux from SM cells at low but not high (~1 vs >6 mM) lactate concentrations[155,156], potentially leading to levels in circulation underrepresenting production at moderate workloads under ketotic conditions.

Thus, the influence of exogenous ketosis on glycolysis, and therefore glycogen (as well IMTAG) utilisation, remain equivocal with limited exploration in postprandial states.

Circulating Glucose

Alongside lowered lactate, a suppression of circulating glucose by exogenous ketosis is well established in fasted[23,107] and postprandial states[23,157], at rest[23,158] and in-exercise[146,159], and in healthy individuals[37] and those with metabolic dysfunction such as T2D[160,161]. The onset of this lowering rapidly proceeds ketosis and is widely suggested to be driven primary by reduced hepatic output rather than increased glucose clearance[23,38,72,160–163].

Plasma glucose lowering has been observed in the absence of alterations to circulating insulin or glucagon[164–166], though insulin and C-peptide have been equivocally found to acutely rise resultant of exogenous ketosis[23,136,167–169]. In humans, KME ingestion has been shown to lower glucose but not affect circulating insulin levels during an OGTT[158,170], whilst a post-exercise feeding study saw no insulin elevation due to the KME[171]. In contrast, under a 2 hr post-exercise 10 mM hyperglycaemic clamp, KME consumption elevated plasma insulin and increased glucose uptake and/or reduced hepatic output, though no effect on insulin was observed prior to the clamp[168], whilst animal studies have reported that small quantities of insulin are released following gavage with exogenous ketones[164,172]. Where insulinotropic effects have been exhibited, current evidence generally supports this being consequent of

greater pancreatic insulin release (alongside lesser release of glucagon, though this is also equivocal[169]) rather than inhibited hepatic/nephrologic breakdown[173–175], with isolated rat pancreatic islets observed to increase secretion when exposed to ketones in a >5 mM glucose media[173]. It has been suggested that βHB drives insulin release through being utilised as an energy source for β-cells and by acting antagonistically on FFAR3[46], though exact mechanisms remain underexplored.

Alongside potential alterations to insulin signalling, ketosis-driven lowering of circulating FAs could act hepatically to increase the sensitivity of glycogenolysis to suppression by hyperglycaemia and drive glucose uptake[158,176], as well as potentially dampen gluconeogenesis through lowered glycerol availability as a substrate[177]. However, ingestion of niacin, which inhibits lipolysis in the same manner as βHB by binding to HCAR2, does not appear to affect glycaemia[178], suggesting glucose lowering may not necessarily be related to FA suppression.

Diminished gluconeogenesis appears to play a role in reducing hepatic glucose output under ketosis, either via reductions in substrate availability or as-yet unexplored direct suppression by KBs[136,179]. Ketone oxidation lowers secretion of the gluconeogenic substrate alanine from extra-hepatic tissue, particularly SM[107,180,181] (1.3.5; *Figure 1.5 Point 5*), explaining 28% of a KME-induced drop in plasma glucose seen in healthy individuals after a 24 hr fast[136]. However, βHB-associated glucose lowering is too rapid to be explained, at least initially[182,183], by reduced gluconeogenesis, and no stable isotope work has been conducted to directly interrogate this effect[184,185].

The glucose-lowering capacity of exogenous ketosis is retained during exercise despite this representing a strong driver for glycaemic homeostasis[134,182,183,186]. In this setting, it appears to remain hepatic-output driven[150,179] and has been established after KME ingestion both fasted[71,107] and postprandially[146,150–152,157], as well as after βHB infusion[72]. Similar to at rest, augmented insulin secretion potentially plays a role[107], though is not universally observed[32,179] and is not well explored. Reflecting this, Howard et al.[150], using methodologies described above, observed an in-exercise fall in hepatic glucose output from 7 to 6.5 g/hr at relatively low βHB levels (~1.25 mM), with glucose clearance and insulin unaffected.

Glucose lowering under exogenous ketosis appears, therefore, to be driven by reduced hepatic output via both insulin-dependent and independent mechanisms. These processes remain poorly characterised and understood however, with hepatic glycogenolytic kinetics yet to be investigated at all. Thus,

mechanistic work, especially within specific metabolic milieus (fed *vs* fasted, rest *vs* exercise, healthy *vs* T2D, etc), is warranted to elucidate drivers and modulators of this effect.

1.3.4 Influence on Protein Metabolism

Ketosis acts anti-catabolically[187], playing a teleologically adaptive role of protecting structural and functional protein during starvation[39]. KBs appear to directly reduce muscle protein catabolism, as evidenced by observations of decreased urinary urea following a prolonged fast[1] and reduced alanine & leucine oxidation following ketone infusion[180,181]. However direct interrogation of the mechanisms at play is lacking in humans[188]. Alongside indirect signalling, KBs supressing glucose oxidation is suggested to indirectly preserve protein stores that would otherwise be catabolised for gluconeogenic amino acids[21]. This is evidenced by exogenous ketosis lowering circulating alanine[107,189], as detailed in *Figure 1.5*, which acts to supress gluconeogenesis[136].

1.3.5 Influence on Sympathetic Tone

βHB binds antagonistically to GPR41 (FFAR3) receptors expressed on sympathetic ganglion, reducing sympathetic tone and consequently reducing EE and heart rate (HR) in mice[190]. This is in contrast to short chain FAs (whose circulating levels are lowered under exogenous ketosis) which activate the same receptors[190], leading to elevated EE and HR[191]. It remains unclear whether sympathetic tone is affected by KME ingestion during acute exercise, potentially influencing central fatigue, cardiac drift, and appetite[192], but exogenous ketosis has been seen to cause overnight suppression in sympathetic tone in an exercise-overtraining human model[193].

1.3.6 Influence on Fat Metabolism

Exogenous ketosis bypasses the metabolic adaptations required for endogenous ketogenesis, thus negating the need for upregulated liberation and hepatic oxidation of FAs. The inverse effect is, in fact, observed with βHB and AcAc acting antilipolytically on adipocytes thus stemming the release of FAs into circulation. This occurs as KBs antagonistically bind to HCAR2 receptors (also known as PUMA-G or NICAR1)[105], inhibiting a pathway which phosphorylates HSL, therefore diminishing its activity[194,195] and slowing flux through the rate limiting step of lipolysis. Accordingly, stable isotope methodologies have reported ~35-50% decreases in lipolysis following βHB infusion[72] and oral ingestion[194], occurring independent of any insulin changes[170].

This reduction in circulating FAs is also present in exercise[53,71,107,146,152,153,157]. Recent untargeted plasma metabolomic analysis revealed reductions in glycerol, long-chain fatty acid, and acyl-carnitine metabolites for KME+CHO compared to CHO-only during steady state running[150]. As FAs are crucial contributors to energy provision during exercise especially at moderate intensities[96], and adipose lipolysis represents a key source[196,197], diminished availability across prolonged exercise may increase reliance on IMTAG for lipid-derived oxidative substrates[198]. It might also act to increase plasma glucose and/or muscle glycogen oxidation[199–204], acting counter to posited ketotic suppression of glycolysis. *In vivo* exogenous ketosis has been seen to increase[107] or not influence[146] IMTAG utilisation during fasted or postprandial exercise respectively, but evidence is limited to these two studies.

Reduced FA availability under exogenous ketosis may consequently impair endurance performance over longer durations, even at intensities with relatively high-carbohydrate dependence[96]. This is evidenced by inhibition of lipolysis via nicotinic acid worsening cycling TT performance in long (120 min) but not shorter duration (60 or 90 min) efforts[205].

1.3.7 Influence of Nutritional State on KME Metabolism

Blood βHB kinetics post-ingestion of the KME appear to be driven by the rates of three factors:

i. Hydrolysis of the monoester bond & uptake into circulation of liberated βHB & 1,3-BD,
ii. Hepatic uptake and conversion of 1,3-BD to βHB, then its release into circulation, and
iii. The disappearance of βHB through ketolysis into AcAc, (potentially) intra-cellular storage, and urinary excretion.

A number of determinants are likely to play differing roles in shaping βHB kinetics in a postprandial state compared to under fasted conditions, where KME metabolism has been well characterised[20,107,110,206]. Rate of gastric emptying is known to slow with ingestion of a high-carbohydrate meal, as well as increasing intestinal transit time[207], which may delay the KME reaching the proximal small bowel for hydrolysis and uptake. MCT saturation for small hydrocarbon uptake in the gut might occur[208], with saturation dynamics themselves affected by luminal pH[209–212] which may be modified by the meal and KME-derived βHB as a weak organic acid. Increased competition for esterases[213] is also possible. Though this appears not to occur at ≤5 mM βHB in circulation or in the liver[121,214], gut esterase kinetics remain unexplored. These could all act to delay breakdown and uptake of βHB and 1,3-BD into circulation. Once in the liver, 1,3-BD may have greater competition for its oxidation to βHB in a postprandial state, especially if alcohol has been consumed with the meal as alcohol dehydrogenase is the rate limiting step for this oxidation[110,121–124,215], further slowing the appearance of KBs into circulation from this pathway. Additionally, any elevations in insulin will depress endogenous ketogenesis[45].

Greater inter-individual variation in βHB kinetics may arise in a fed, compared to a fasted, state through interactions between the nutrients consumed and variance in expression of isoforms of gut esterases[216], the expression[217] & distribution of MCTs (apical: MCT1; basolateral: MCT4 and MCT5)[218,219], and the content/activity of alcohol and aldehyde dehydrogenases in the liver[124,220]. Additionally, metabolic responses to feeding itself, such as elevations in insulin, are known to exhibit intra-individual, in addition to inter-individual, variability[221].

Alongside βHB appearance into circulation being influenced by nutritional state, disposal rates may also be affected. A reduced rate of conversion of βHB to AcAc consequent of meal-induced reductions in the hepatic NAD^+/NADH ratio is hypothesised to occur[23,123,222], whilst oxidation would likely also be reduced through substrate competition if the meal was high-carbohydrate in nature (1.4.2)[223]. Increased postprandial circulating insulin levels have been suggested to elevate βHB clearance rates into extra-hepatic tissue, although no clear mechanism has been evidenced[23,168,224]. Furthermore, it is unclear if ketosis itself potentiates, or directly stimulates, the secretion of insulin, in either a fed or fasted state, and what feedback effects this may have on βHB metabolism. Intra-cellular storage of ketones has also been previously indicated[107], but never directly investigated, so the influence on this of other macronutrient availability is unclear.

It might therefore be expected that in a fed state, compared to a fasted one, there will be a slower time to peak, and lower peak concentration, for βHB in the bloodstream, but with a prolonged duration of ketosis. Alongside this, as the glucose lowering effect of exogenous ketosis appears to be driven by supressed hepatic release, the magnitude of this effect may be diminished postprandially as a greater proportion of circulating glucose would have an exogenous source and hepatic output would already be supressed, compared to fasted where glycogenolysis/gluconeogenesis represents the primary source of plasma glucose[225,226]. Similarly, reductions in circulating FAs concentrations by ketosis may be lessened due to both exogenous lipid sources reducing the proportional impact of altered endogenous supply, and ketone-independent suppression of lipolysis by elevated insulin reducing the scope for further inhibition[227]. Taken together there is a clear need to explore how βHB kinetic profiles, and the wider metabolic effects of exogenous ketosis, might differ under different nutritional states, particularly in the as-yet uncharacterised context of KME ingestion proceeding a substantial high-carbohydrate meal as would be best practice for performance-oriented endurance exercise.

1.4 Exercise Responses to Exogenous Ketosis

1.4.1 Exercise Performance

In work by Cox et al.[107], ingestion of the KME improved fasted cycling TT performance by ~2% compared to placebo. These observations are suggested to have launched the concept of exogenous ketosis as an ergogenic aid for exercise within both research and applied sports science[228]. Subsequent findings, however, have been equivocal with limited evidence for the KME holding ergogenic properties, including a study that carried a similar protocol to that of Cox et al.[107] but did so postprandially in runners and found no effect compared to placebo[154]. In fact, a number of studies have found detrimental impacts of KME ingestion on performance[150,152,229,230]. The only works to have reported a benefit in a fed state were those conducted within the context of rugby match-simulation[231] (where the outcome carried cognitive as well as physical determinants), when economy was the outcome measure[232] (though this finding wasn't subsequently replicated by the same group[233]), or when sodium bicarbonate was co-ingested with the KME to buffer ketoacidosis (1.4.4)[157,234]. However, with reference to the latter, interactions between multiple nutritional supplements are beyond the scope of this thesis.

The equivocal nature of findings may be in part explained by heterogeneity in testing protocols such as the exercise intensities, durations, and modalities used, the athlete populations (age/training-status/sex) studied, provision of other macronutrients prior to and during exercise, and the circulating [βHB] reached due to the KME dosing levels/timings used[24,31,65]. *Table 1.5* summarises the current literature pertaining to KME use for exercise performance. It is limited to endurance or endurance-related outcomes as there is little rationale, nor evidence, for exogenous ketosis being beneficial for high intensity/intermittent exercise[31,65,235,236]. The exercise-related elements of this thesis therefore also focus on endurance performance.

***Table 1.5* - Summary of studies where the influence of acute KME ingestion on endurance, or endurance-related ($\dot{V}O_{2peak}$/W_{max}/economy/efficiency), performance has been explored.**

Study	Participants	Exercise Protocol/s	Dosing	Study Design	Performance Outcome/s
Cox et al. (2016) [107]	6 M / 2 F Endurance athletes $\dot{V}O_{2max}$ M: 5.37 ± 0.3; F: 3.30 ± 0.1 L/min	*Cycling (ergometer)* 60 min @ 75% W_{max} 30 min TT	KME 573 mg/kg, isocaloric to PLA CHO @ 1.2 g/min (KME condition)	*Randomised:* Y *Crossover:* Y *Double Blinding:* Unclear *Fuelling State:* Fasted	#TT distance (m): KME 411 ± 162 m (mean ± SEM) further vs PLA
Evans & Egan (2018)* [151]	11 M Competitive team sport athletes $\dot{V}O_{2peak}$ 53.9 ± 2.2 ml/min/kg	*Running (LIST)* 5 × 15 min intermittent 20 m shuttle to exhaustion	KME 750 mg/kg, not isocaloric to PLA CHO @ 1.2 g/min	*Randomised:* Y *Crossover:* Y *Double Blinding:* Y *Fuelling State:* Fed (3 g/kg CHO)	TTE (s): KME 229 ± 72 PLA 267 ± 96
Evans et al. (2019) [154]	7 M / 1 F Middle/long distance runners $\dot{V}O_{2peak}$ 62.0 ± 5.6 ml/min/kg	*Running (treadmill)* 60 min @ 65% $\dot{V}O_{2max}$ 10 km TT	KME 573 mg/kg, not isocaloric to PLA CHO @ 1 g/min	*Randomised:* Y *Crossover:* Y *Double Blinding:* Y *Fuelling State:* Fed (2 g/kg CHO 2 hr prior)	TT time to complete (s): KME 2402 ± 237 PLA: 2422 ± 246
Dearlove et al. (2019) [153]	9 M / 3 F Healthy athletes $\dot{V}O_{2max}$ 4.4 ± 0.2 L/min	*Cycling (ergometer)* Step test to exhaustion	KME 330 mg/kg, non-caloric PLA No CHO	*Randomised:* Y *Crossover:* Y *Double Blinding:* Single *Fuelling State:* Fasted	W_{max}: KME 393 ± 22 PLA 389 ± 20
Poffé et al. (2020) [146]	12 M Trained cyclists $\dot{V}O_{2max}$ 62.4 ± 6.6 mL/min/kg	*Cycling (turbo/ergometer)* 180 min varying intensity 15 min TT 'Sprint' @ 175% LT	KME 65 g (918 ± 102 mg/kg), not isocaloric to PLA CHO @ 1.2 g/min	*Randomised:* Y *Crossover:* Y *Double Blinding:* Y *Fuelling State:* Fed (~ 2600 kJ; 72% CHO)	TT (W): KME 273 ± 38 PLA 272 ± 37 Sprint TTE (s): KME 59 ± 16 PLA 58 ± 17

Study	Participants	Exercise Protocol/s	Dosing	Study Design	Performance Outcome/s
Poffé et al. (2021) [237]	9 M Trained cyclists $\dot{V}O_{2max}$ 61.0 ± 2.9 mL/min/kg	*Cycling (turbo/ergometer)* 180 min varying intensity 15 min TT 'Sprint' @ 175% LT	KME 65 g (922 ± 85 mg/kg), not isocaloric to PLA CHO @ 1.2 g/min	*Randomised:* Y *Crossover:* Y *Double Blinding:* Y *Fuelling State:* Fed (~ 2600 kJ; 72% CHO)	TT (W): KME ~254, PLA ~254 Sprint TTE (s): KME 55 ± 19 PLA 55 ± 21
Poffé et al. (2021) [152]	12 M Highly trained cyclists $\dot{V}O_{2max}$ 62.5 ± 5.5 mL/min/kg	*Cycling (turbo/ergometer)* 60 min warm up 30 min TT 'Sprint' @ 175% LT	KME 50 g KME (726 ± 75 mg/kg), not isocaloric to PLA 60 g CHO during warm up	*Randomised:* Y *Crossover:* Y *Double Blinding:* Y *Fuelling State:* Fed (~ 2600 kJ; 72% CHO)	[#]TT (W): 3.8 ± 1.5 W greater in PLA than in KME Sprint TTE (s): No difference
McCarthy et al. (2021) [125]	10 M / 9 F Endurance trained $\dot{V}O_{2max}$ 62.5 ± 5.5 mL/min/kg	*Cycling (ergometer)* 30 min @ VT1 (71 ± 3% $\dot{V}O_{2peak}$) 3 kJ/kg TT	KME 600 mg/kg, not isocaloric to PLA 25 g CHO 35 min prior to exercise	*Randomised:* Y *Crossover:* Y *Double Blinding:* Y *Fuelling State:* Fed (1 g/kg CHO)	TT (W): KME 196, PLA 201
Peacock et al. (2022) [231]	9 M Professional rugby players	*Running (rugby pitch)* Simulated rugby union match protocol (BURST) High intensity performance, 15 m sprint, & sled push tests	KME 590 mg/kg, isocaloric to PLA KME: 20 ± 2 g CHO PLA: 90 ± 9 g CHO Split 2:1 pre- & mid-exercise	*Randomised:* Y *Crossover:* Y *Double Blinding:* Y *Fuelling State:* Fed *(habitual breakfast, ~165 g CHO)*	[#]Time to complete for high intensity performance test (s): 0.33 ± 0.41 (2.1%) quicker in KME vs PLA Sprint & sled push times (s): No difference
Waldman et al. (2022)* [238]	14 M Professional firefighters	*'Live-burn search & rescue'*	KME 500 mg/kg PLA non-caloric	*Randomised:* Y *Crossover:* Y *Double Blinding:* Y *Fuelling State:* Fasted	Time to complete (min): KME 10.6 ± 0.6 PLA 10.6 ± 0.8

Study	Participants	Exercise Protocol/s	Dosing	Study Design	Performance Outcome/s
Howard et al. (2023) [150]	16 M / 1 F Healthy recreationally active $\dot{V}O_{2peak}$ 4.1 ± 0.5 L/min	*Running (treadmill)* 90 min (54 ± 3% $\dot{V}O_{2peak}$) with weighted vest (30% body mass), then TTE without vest @ 85% $\dot{V}O_{2peak}$ *Separate day* - 6.4 km TT (1% gradient) with weighted vest	KME 573 mg/kg, not isocaloric to PLA 110 g glucose @ ~ 30 g/hr (90 min/TTE) or pre-exercise (TT) Glucose infusion (90 min/TTE) 0.78 µmol/kg/min	*Randomised:* Y *Crossover:* Y *Double Blinding:* Y *Fuelling State:* Fasted	#TTE (s): 104 (10%) greater in PLA than in KME #TT (s): 141 (4.1%) faster in PLA than in KME
McCarthy et al. (2023) [230]	11 M / 4 F Endurance trained $\dot{V}O_{2peak}$ 60 ± 9 mL/min/kg	*Cycling (ergometer)* 30 min @ VT1 (67 ± 4% $\dot{V}O_{2peak}$) Step test to exhaustion	KME 600 mg/kg, not isocaloric to PLA	*Randomised:* Y *Crossover:* Y *Double Blinding:* Y *Fuelling State:* Fasted	#W_{max}: KME 359 ± 61 PLA 375 ± 64 No difference in sub-max. & peak cardiac output (Q_c)
McCarthy et al. (2023) [229]	21 M / 2 F Trained cyclists $\dot{V}O_{2peak}$ 65 ± 12 mL/min/kg	*Cycling (ergometer)* 15 min warm up 20 min TT	KME 350 mg/kg, not isocaloric to PLA	*Randomised:* Y *Crossover:* Y *Double Blinding:* Y (blinded analysis) *Fuelling State:* Fasted	#TT (W): 2.4% lower in KME vs PLA KME 255 ± 54 PLA 261 ± 54
Waldman et al. (2023)* [239]	12 F 'Trained' and 'Elite' athletes[79] $\dot{V}O_{2peak}$ 34.3 ± 3.7 mL/min/kg	*Cycling (ergometer)* 6x5 min 40% to 65% W_{max} in 5% intervals 10 km TT	KME 375 mg/kg, not isocaloric to PLA 1 g/min CHO	*Randomised:* Y *Crossover:* Y *Double Blinding:* Y *Fuelling State:* Fasted	TT (min): KME 29.7 ± 5.7 PLA 29.6 ± 5.7

Study	Participants	Exercise Protocol/s	Dosing	Study Design	Performance Outcome/s
Brady & Egan (2023) [232]	11 M Middle & long distance runners $\dot{V}O_{2peak}$ 59.4 ± 5.9 mL/min/kg	*Running (treadmill)* 8 min @ five submaximal speeds (10–14 km/hr) Ramp test to exhaustion	*3x Conditions* KME only KME+CHO CHO only KME 750 mg/kg, not isocaloric to PLA 1 g/min CHO (maltodextrin)	*Randomised:* Y *Crossover:* Y *Double Blinding:* Y *Fuelling State:* Fed (~8.5 kcal/kg, ~1.2 g/kg CHO >3 hr prior)	[#]Running Economy: 4.1% lower in KME vs CHO Not different between KME+CHO & CHO TTE (s): KME 333 ± 106 KME+CHO 342 ± 99 CHO 369 ± 116
Bone et al. (2025) [94]	16 M / 12 F Endurance exercise trained $\dot{V}O_{2peak}$ 59 ± 11 mL/min/kg	*Cycling (ergometer)* 5 min @ 75%, 100%, 125% VT1 Ramp test to exhaustion	*3x Conditions* High KME 600 mg/kg Low KME 300 mg/kg PLA	*Randomised:* Y *Crossover:* Y *Double Blinding:* Y *Fuelling State:* Fed (habitual diet)	Gross economy/gross efficiency/delta efficiency/$\dot{V}O_{2peak}$: Not different between conditions [#]W_{max}: Lower for High KME (329 ± 60) than Low KME (339 ± 62) & PLA (341 ± 61)
Brady et al. (2025)* [233]	10 M / 8 F Middle & long distance runners $\dot{V}O_{2peak}$ 59.4 ± 7.2 mL/min/kg	*Running (treadmill)* 8 min @ five submaximal speeds (10–14 km/hr) Ramp test to exhaustion	*2x Conditions* KME only (no CHO) PLA as CHO only KME 750 mg/kg, not isocaloric to PLA PLA 1 g/min CHO (maltodextrin)	*Randomised:* Y *Crossover:* Y *Double Blinding:* Y *Fuelling State:* Fed (~8.5 kcal/kg, ~1.2 g/kg CHO >3 hr prior)	Running Economy: No difference between KME & PLA TTE (s): KME 329 ± 131 CHO 356 ± 140

*All studies conducted under normoxia. Any conditions/arms where KME was co-ingested with another ergogenic supplement (e.g. sodium bicarbonate, caffeine) are excluded as this thesis concerns the isolated effects of the KME. BURST, Bath University Rugby Shuttle Test; CHO, carbohydrate; F, female; LIST, Loughborough Intermittent Shuttle Test; M, male; TT, time trial; TTE, time-to-exhaustion; Q_c, cardiac output; VT1, ventilatory threshold 1. *, KE4 (KetoneAid) KME used, otherwise the KME was deltaG (TdeltaS) - both contain the KME, but with a different production method and additives/flavourings. Any metric presented relative to kg ('/kg') is scaled onto bodyweight. [#] $p < 0.05$ between KME and PLA/control. All data presented as mean ± SD unless otherwise stated.*

1.4.2 A Low-Carbohydrate State to 'Unlock' Ketone Oxidation?

Common across studies that have seen a benefit of exogenous ketosis to endurance performance, as a TT[107,231] or as a performance determinant such as efficiency or economy[71,232], is lowered carbohydrate, primarily glycogen, availability ('low-carbohydrate'), through fasted exercise[71,107] and/or sub-optimal in-exercise carbohydrate provision[71,107,231,232]. *Ex vivo* observations by Petrick et al.[223] found that βHB was able to drive mitochondrial respiration within SM fibres when pyruvate was sub-saturating but not saturating. Therefore, it is plausible that, when abundant, glucose outcompetes KBs as an oxidative substrate but not when availability is constrained. Thus, a low-carbohydrate state might be required to 'unlock' substantive KB oxidation rates and therefore contribution to EE, especially during exercise[31].

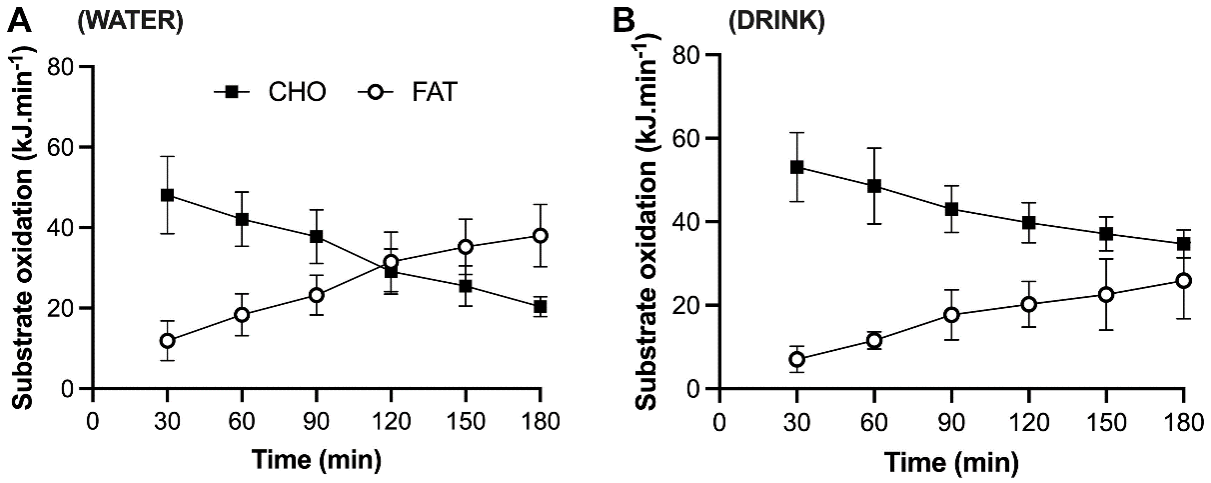


***Figure 1.6* - Substrate oxidation rates from carbohydrate and fat across 180 min of cycling exercise at 95% lactate threshold 1 power (upper-moderate intensity domain).**
A, water only; B, 120 g/hr carbohydrate (CHO) given as a drink. Adapted from Hearris et al.[240].

A fasting-induced low-carbohydrate state, as has been employed in a number of studies[70,71,150,153,229,230,238,241], does not reflect the nutritional state under which athletes typically compete[3,242]. An alternative low-carbohydrate state can be found when liver and SM glycogen stores are depleted across multiple hours of postprandial exercise[134], a phenotype much more commonly found in competition, and thus carrying greater ecological validity for exploration of ketotic influence. This depletion is largely considered obligate, occurring even when exogenous carbohydrate is provisioned and oxidised at maximal rates of ~1.5 g/min[144,240,243], as in the upper-moderate intensity domain well-

trained athletes might be oxidising >3 g/min total carbohydrate[144,240,243–245]. This results in a potential net deficit of >1.5 g/min that increases proportional to intensity[96] and must be met by endogenous carbohydrate utilisation. Functioning to spare progressively depleted glycogen stores during this prolonged exercise, the contribution of carbohydrate oxidation to EE diminishes with time[5,134,240,246] (*Figure 1.6*), likely mediated by blunted PDH flux through reduced pyruvate availability and increased PDH kinase activity[203,246]. To meet metabolic demands, lipid oxidation increases[247] contributing to economy worsening after ~2 hr of exercise[248,249] due to β-oxidation's inferior P/O (*Table 1.3*).

As detailed, alongside this exercise-induced reduction in carbohydrate availability potentially comes increased capacity for KB oxidation. Crucially, in a low-carbohydrate setting where exogenous carbohydrate is still provisioned, pyruvate supply, though diminished, should still be sufficient to anaplerotically replenish oxaloacetate within the TCA cycle. This is posited to be necessary for high KB oxidation rates[60,70,107] though this relationship, and its importance in the oxidative substrate hierarchy, remains debated[137,179,223]. Thus, a low-carbohydrate state can be achieved across multi-hour exercise, with exogenous carbohydrate intake meeting both best practice for fuelling[3] and anaplerotic needs for ketolysis, potentially establishing a 'best of both worlds' scenario for ketone oxidative capacity.

1.4.3 Exercise Efficiency & Economy

Exercise efficiency describes the ratio between external work-rate (power output during cycling, speed during running, etc) to the total EE required to match this work-rate[250]. A commonly employed alternate index of efficiency is economy, the ratio of external work-rate relative to total oxygen cost ($\dot{V}O_2$ *per* work-rate)[251,252]. Efficiency metrics are conventionally applicable at intensities within the moderate and heavy domains where $\dot{V}O_2$ is able to stabilise, a metabolic steady state is achievable, and anaerobic work capacity isn't being progressively depleted[252,253], and have been best characterised in cycling due to the ease of calculating power and standardising conditions[252]. They can be expressed in two forms: 1. 'gross' representing the whole body, or 2. 'delta' reflecting the isolated active musculoskeletal system (though validity of this interpretation is debated)[250–252,254]. Both have been evidenced as predictors of moderate/heavy intensity performance[95,249,250,255] and are responsive to nutritional interventions, e.g. dietary nitrate[256].

Exogenous ketone oxidation invalidates calculation of EE from indirect calorimetry (discussed further in 2.3.6), thus efficiency (when expressed as external work rate relative to EE) cannot be confidently measured under a state of exogenous ketosis[69,257]. Economy remains validly quantifiable, however, and could potentially be improved by βHB's superior Gibbs' free energy (ΔG′) redox span compared to glucose, and ~40% greater P/O than for FAs. Dearlove et al.[70] showed, utilising ^{13}C-labelled KME, that ~0.28 g/min βHB oxidation contributes up to ~8.4% to total EE during moderate exercise in a depleted-glycogen (i.e. low-carbohydrate) state, thus ketolysis can tangibly contribute to energetic provision in exercise. If this ketolysis were to supplant any energetic provision from β-oxidation, which could be depressed by lower circulating FA availability under ketosis[105,258], then one might expect a reduction in oxygen requirement for a given workload, i.e. an improvement in economy. Accordingly, exercise economy benefits have been seen under exogenous ketosis, though studies are limited[71,232].

Given the relatively minor (if still tangible) contributions to EE of βHB oxidation that tracer studies have observed however[70,71], enhanced oxidative efficiency is unlikely to explain the entirety of these improvements. Though less well explored, alterations in plasma calcium and sodium have been reported under exogenous ketosis[146,152]. These could speculatively influence neuromuscular transmission and sarcoplasmic reticulum (SR) calcium handling, critical for actin-myosin crossbridge formation, thus potentially muscle contractile efficiency[259,260].

1.4.4 Ketoacidosis & Cardiorespiratory Measures

As βHB and AcAc are weak organic acids, ketosis is characterised by a mild and transient state of ketoacidosis. Metabolic compensation occurs via depletion of circulating bicarbonate (HCO_3^-) which buffers excess H^+, with resultant CO_2 offloaded in breath[261]. This co-reduction in pH and HCO_3^- has been observed under exogenous ketosis both at rest[23,262] and in-exercise[71,152,153,157], with a consistent pH drop of ~0.05-0.10. Acidosis, and subsequent loss of extracellular buffering capacity, is associated with impaired exercise performance especially at high workloads[263], though evidence is more equivocal at moderate intensities[264]. Co-ingestion of sodium bicarbonate with the KME to increase the concentration, thus buffering capacity, of circulating HCO_3^-[265], has recently been investigated as a strategy to attenuate

this ketoacidosis. Indications are that it might improve exercise performance compared to KME alone during exercise >~1 hr[152,157,266], but research outcomes are too limited to draw solid conclusions.

The mechanisms linking elevated H^+ to premature SM fatigue aren't fully elucidated and the magnitude of effect is debated[267–269], but acidosis is known to disrupt contractile efficiency[270]. It does so through inhibiting ADP isomerization and the low- to high-force cross-bridge transition[271]. Lowered pH also reduces myofilament sensitivity to Ca^{2+} via competition with H^+[270,272,273], alongside being suggested to slow Ca^{2+} release from the SR[260,274], with both acting to reduce the rate at which Ca^{2+} binds to troponin C and therefore the rate of cross-bridge detachment. This in turn slows shortening velocity, a key determinant of muscular efficiency[252]. Additionally, acidosis inhibits key glycolytic enzymes such as glycogen phosphorylase and PFK[275–277], whilst phosphorylcreatine resynthesis is also slowed[275]. Mitochondrial ATP resynthesis function is potentially also impaired through elevated content and activation of UCP3[278]. Thus, under acidosis both oxidative phosphorylation, and force generation utilising the ATP it produces, may become less efficient.

Modulation of intra- and extra-cellular ion distributions might also be implicated in the ergolytic effect of acidosis with, most prominently, lowering of potassium at a SM level established as detrimental[259,260]. However, whilst plasma hypokalaemia has been seen under exogenous ketosis at rest[23], it's not been observed during exercise[152,157]. In contrast, there is evidence that at high exercise intensities circulating calcium, sodium, and chloride are elevated after KME ingestion[152], with calcium and sodium, but not chloride, elevated at lower intensities[146]. These findings are not universal[157], though, whilst the implications on exercise of electrolyte imbalance in isolation of hypohydration are not well established[279,280], especially as circulating levels don't necessarily reflect those within working muscle.

Beyond circulating metabolites, acidosis also acts to increase ventilatory rates in exercise. As per the Henderson-Hasselbach equation, additional H^+ in circulation will act to raise blood pCO_2, with both this and elevated $[H^+]$ itself stimulating chemoreceptors to increase ventilatory drive[281–284]. Though an increase in pCO_2 is not universally elicited under exogenous ketosis[262], elevations to minute ventilation (V_E) have been observed after KME ingestion during 30 min of cycling at VT1 (+6 L/min compared to PLA)[125], 3 hr 'intermittent' intensity cycling (+9 L/min)[157], and at maximal but not submaximal intensities during a cycling ramp test (+8 L/min)[153], with plasma βHB at 2-4 mM in all instances. In a

well powered study, McCarthy et al.[230] found that 33% of the variance seen in V_E was explained by pH in an inverse relationship. As this leaves 67% of the variance unexplained, and this relationship hasn't been universally observed[94], other factors are likely at play in increasing respiratory drive under ketosis. Furthermore, work by Faull et al.[285] found that 'anxiety of breathing' was elevated under exogenous ketosis during a cycling ramp test, with pH and plasma [βHB] both predictors of this response, indicating that ketoacidosis plays a direct role in increasing perceived ventilatory drive during exercise. V_E itself was not, though, a predictor of any RPE measure reported in their study, despite two of the five measures being ventilation-related, again indicating that the interactions present are multi-faceted. It is also possible, for example, that increased ventilation acts to counter elevated blood acetone concentrations[286], though this mechanism and its influence remains underexplored.

As elevated ventilation carries an additional energetic, and therefore oxygen, cost within pulmonary musculature[282], one might expect concurrent elevations in HR to meet these demands[94,283,284]. The impact of exogenous ketosis on exercising HR is mixed however, with studies reporting elevations[125,229,230,287], no effect[151–153,237], or a reduction[232] compared to placebo, and with no clear patterns present explaining the variance in this relationship. Where HR increases have been observed, suggested mechanisms include increased circulating noradrenaline[287] and activation of bitter taste-receptors on cardiac tissue[288], or nicotinic acid receptors[45,289]. Despite findings of a 3-4 bpm elevation in sub-maximal HR, McCarthy et al.[266] established no effect of the KME on cardiac output, and without acidosis or V_E being a predictor. Thus, where equivocally present, elevations in HR with the KME appear to be accompanied by reductions in stroke volume and may not be directly linked to ventilatory costs.

1.4.5 Gastrointestinal Disturbances

Whilst the KME is generally better tolerated than other EKSs[31] (*Table 1.4*), gastrointestinal (GI) disturbances after ingestion have been reported in existing work[125,151,152,231]. Though not universally present[159], symptoms include flatulence, diarrhoea, cramping, belching, heartburn, and nausea. A recent study observed increased abdominal discomfort and elevated circulating intestinal fatty acid-binding protein (I-FABP), a biomarker of intestinal barrier dysfunction, under KME+CHO compared to CHO-alone, though a similar biomarker claudin-3 was unaffected and neither GI symptoms nor these

biomarkers were associated with performance outcomes when the KME was found to be ergolytic[150]. As GI upset can though detrimentally influence exercise performance[111], current best practice is to split KME doses into smaller boluses given >30 min apart and for them to be provisioned prior to, rather than during, exercise where possible[31,94,157].

1.4.6 Appetite

Given ketones' evolutionary role as cerebral metabolic fuel in times of energetic crisis[39,85], it is understandable that they might influence appetite signalling, potentially tempering deleteriously high desire to eat at times when food is most scarce. Certainly, intracerebroventricular infusions of βHB decreased energy intake (EI) and bodyweight in rats[290], though, aside from hypothalamic involvement[45,291], the mechanisms underpinning this remain unclear.

Beyond central effects, ketosis appears to influence secretion of appetite/satiety hormones. Circulating leptin is elevated in rodents fed both a ketogenic diet[292] and the KME[293], whilst ghrelin and appetite have been shown to be supressed in humans at rest after KME ingestion with a putative mechanism of βHB antagonising GPCR-41, a Gi/Go protein-coupled receptor, on gut enteroendocrine cells[162]. Post-exercise, exogenous ketosis has been shown to reduce plasma ghrelin levels in humans, correlating with supressed hunger perception and desire to eat, with leptin unchanged[146]. Alterations to circulating hormones doesn't universally translate to changes in appetite in an exercise setting, however. Okada et al.[294] observed that KME ingestion lowered post-exercise ghrelin and GLP-1 but did not influence appetite nor *ad libitum* EI, whilst no hunger hormones nor appetite were affected during, or post, prolonged intermittent cycling exercise[237]. It is also suggested that ketoacidosis might supress appetite, independent of hormonal signalling, as KME-induced post-exercise reductions in appetite were abolished by sodium bicarbonate co-ingestion[157], though the mechanism is unclear. Longer term, exogenous ketosis was observed to reduce the rate at which GDF15, a stress-induced hormone and potential exerkine known to supress appetite[295,296], rose across a three week human exercise-overtraining protocol leading to increased EI compared to the control group[193]. It could therefore be posited that, in an exercise setting, KBs might acutely supress appetite through lowered ghrelin, but conversely increase it mid/long term through reductions in GFD15.

1.5 Exogenous Ketosis & *De Novo* Lipogenesis

1.5.1 *De Novo* Lipogenesis

The liver plays a central role in regulating whole-body FA metabolism, transitioning between storage and secretion states in response to different metabolic conditions[297]. A process key to maintenance of lipid homeostasis, conserved across mammalian physiology[298], is *de novo* synthesis of FA, or *de novo* lipogenesis (DNL). This predominantly takes place hepatically, within pericentral regions of the liver where reduced oxygen availability lowers β-oxidation flux, preventing futile cycling[299]. During DNL, excess non-lipid precursors, primarily glucose, as well as fructose, lactate, amino acids, and acetate, are converted to saturated and mono-unsaturated FAs[196]. These can be oxidised or esterified with glycerol hydroxyls within the endoplasmic reticulum for intrahepatic storage as triacylglycerol (IHTAG) in lipid droplets or secretion into circulation within very low density lipoproteins (VLDL) for delivery to peripheral tissue[196]. These lipids can then be used for membranes, signalling, extra-hepatic storage, and energy provision[57]. Though DNL can also take place in adipose tissue, hepatic DNL is thought to act more efficiently and, in healthy individuals, contributes more to circulating triacylglycerol (TAG)[300]. Despite being an energetically costly process, requiring significant ATP, Ac-CoA, and NADPH provision for each condensation reaction[298], it adaptively serves to convert an 'energy excess' of substrate, whose storage capacity might be limited, into lipids, whose storage capacity is functionally infinite[301]. DNL is therefore a tightly controlled process that can be influenced by a number of energy state-dependant hormones, primarily insulin[302], plus potentially leptin[303] and glucagon[304,305], as well as substrate availability, principally glycolytic intermediates[306], and AMPK signalling[307]. This regulation occurs both acutely in response to energy availability[298], and chronically with lipogenic gene expression modulated by sterol regulatory element-binding protein-1c (SREBP-1c) and carbohydrate-responsive element-binding protein (ChREBP)[308].

During DNL when monosaccharides, lactate, or amino acids are the lipogenic substrate, citrate accumulates in mitochondria under energy excess, exits the TCA cycle, and enters the cytosol through mitochondrial citrate carriers (CIC; 'citrate-fed' DNL). There it is converted to Ac-CoA by ATP-citrate lyase (ACLY), which is activated by insulin[309]. This Ac-CoA is in turn carboxylated into malonyl-CoA by Ac-CoA carboxylase (ACACA), a process inhibited by high levels of palmitoyl-CoA[310], with the

rate-limiting enzyme fatty acid synthase (FASN) converting this malonyl-CoA into the sixteen carbon palmitate by first initiating a new acyl chain then elongating it with further malonyl-CoA[196,311–313]. Palmitate, a saturated FA, is thus the primary product of DNL, though stearate and other FAs are also produced if alternative forms of CoA are utilised and/or if post-DNL modification takes place[314], with both palmitate and stearate able to be desaturated by stearyl-CoA desaturase (SCD), yielding palmitoleate and oleate, respectively[315].

Though DNL only produces 1-2 g/day of FAs in healthy individuals[306], compared to an average dietary fat intake of 60-90 g/day[316], it can be elevated up to ten-fold during a high-carbohydrate diet[301,317,318], such as one consumed by endurance athletes, and is chronically upregulated in a number of metabolic disease states[300,302,319]. As hepatic lipid storage capacity is limited[311], prolonged elevation of DNL is linked to liver fat accumulation and therefore the pathogenesis of metabolic dysfunction-associated steatotic liver disease (MASLD; formerly NAFLD)[320–323]. Alongside this, the proportion of IHTAG that is saturated positively relates to DNL and negatively correlates with hepatic insulin sensitivity[324], whilst DNL-associated increases to the saturation and size of the plasma TAG pool are suggested to contribute to atherogenic lipoprotein phenotypes[319].

1.5.2 Potential Influence of Exogenous Ketosis

The impact of exogenous ketosis on lipogenic pathways has not yet been directly explored. Whilst KME ingestion robustly reduces circulating glucose concentrations[37], thus its availability as the primary lipogenic substrate[325] which might supress lipogenesis, exogenous ketones have also been equivocally observed to increase insulin levels, which would act to increase DNL[302,326], whilst AcAc itself is a lipogenic substrate[48,57]. It is therefore unclear whether KBs might influence DNL, and if they do whether lipogenic rates might be up or down regulated.

Within the setting of metabolic health, EKSs are, at present, primarily being studied in humans as an adjuvant therapy for T2D based on their ability to modulate glycaemia[163,327–329]. Metabolic diseases are multifaceted, however, with aberrant hepatic lipid accumulation and metabolism key drivers of insulin resistance[319]. Reflecting this, in 2019 over 55% of patients with non-alcoholic fatty liver disease

(NAFLD), which carried a worldwide prevalence of 37%, also had T2D[330]. If ketosis was able to lower DNL in these disease states, thus reducing IHTAG accretion, then it might have the potential to break the proposed 'twin cycle'[331] of positive feedback between ectopic liver, consequently pancreatic, TAG accumulation and insulin resistance, alongside acutely controlling hyperglycaemia[332] and prospectively also reducing cardiovascular disease risk[333].

Whilst the impact of exercise on DNL itself is equivocal[334–337], it is commonly recommended as an intervention to slow/reverse the onset of liver fat accumulation[337]. Therefore, the potential influence of exogenous ketosis on lipogenesis also carries relevance in exercise settings as it might act to complement, or blunt, the metabolic health benefits of physical activity[338]. Additionally, ketotic modulation of DNL could affect athletic performance, given that glucose and lipid metabolisms are heavily interdependent and the central role the liver plays in maintaining blood glucose homeostasis[41,183], especially during exercise[132,258,339–341]. Thus, in athletes consuming a high-carbohydrate high-sugar diet[5,227], which is known to upregulate DNL[321,342,343] and therefore potentially increase the scope for modulation by ketosis, modified flux through lipogenic pathways may alter oxidative substrate availability pre-/in-exercise.

Exploration of the potential interactive relationship between DNL and exogenous ketosis may consequently inform when, and if, the KME is recommended for both healthy individuals and those with metabolic disorders, within the context of both exercise and broader health.

1.6 Thesis Aims

Across three studies, this thesis endeavours to explore the effects of KME-induced exogenous ketosis on human physiology and metabolism, both in exercise and at rest. Findings might deliver novel insights into where this unique metabolic state may be best applied in both athletic and therapeutic settings.

It has three specific aims:

Aim 1

To investigate if ingestion of the KME, in a dose-response manner, elicits multimodal endurance exercise performance benefits in a high-carbohydrate postprandial setting.

Aim 2

To determine if exogenous ketosis influences cycling economy, and subsequent high intensity work capacity, whilst in a glycogen depleted (low-carbohydrate) state achieved through prolonged moderate intensity exercise.

Aim 3

To examine whether exogenous ketosis modulates postprandial hepatic DNL in healthy individuals, and to characterise how KME ingestion influences circulating metabolism in fed and fasted states.

Chapter 2 - General Methods

2.1 Ethical Approval

All studies reported in this thesis were reviewed and approved by the University of Oxford Central University Research Ethics Committee (CUREC) as part of the following applications:

- *R73636/RE005* (*Chapter 3*)
- *R82053/RE002* (*Chapters 4 & 5*)

2.2 Participants

Participants were recruited using posters circulated on social media (Facebook/Instagram; *all Chapters*) and emailed to local sports clubs (*Chapters 3 & 4 only [exercise relevant chapters]*).

Inclusion criteria common to all studies: aged 18-65 yr, fluent in English, no existing health conditions or food allergies incompatible with the study, non-smoker, consuming a mixed macronutrient diet (no ketogenic diet within the previous 6 months), BMI ≤30 kg/m^2, not currently a participant in any other exercise or nutritional intervention study; female-only (self-identifying): not pregnant, not currently breastfeeding, pre-menopausal, not undertaking hormone replacement therapy (HRT).

Inclusion criteria specific to *Chapters 3 & 4*: performance cycling and running (*Chapter 3*)/cycling (*Chapter 4*) training experience of ≥ 2 yr, $\dot{V}O_{2peak}$ ≥ 55 (male)/ ≥ 45 (female) mL/kg/min, habitual diet adheres to current sport nutrition principles[3,5]. All athletes in *Chapters 3 & 4* were therefore classified as Tier 2 (*Trained/Developmental*) or Tier 3 (*Highly Trained/National Level*) per the classification framework proposed by McKay et al[79].

Inclusion criteria specific to *Chapters 5*: no known history of moderate-to-severe motion sickness, physically active (≥30 min/day, ≥3 days/week).

2.3 Testing Procedures

Each study consisted of a Baseline visit followed by multiple Experimental testing visits (*Chapter 3*: three visits; *Chapter 4*: two visits; *Chapter 5*: four or five visits), all in person. *Chapter 3* also included a virtual Screening Call prior to the Baseline visit.

In-person visits took place in the Department of Physiology, Anatomy, & Genetics (DPAG; *Chapter 3*) and the Clinical Research Unit (CRU) of the Oxford Centre for Diabetes, Endocrinology, & Metabolism (OCDEM; *Chapters 4 & 5*), both University of Oxford. *Chapter 3* & 4 visits were undertaken in a climate-controlled exercise physiology laboratory (18-20°C, 35-55% humidity). *Chapter 5* visits took place in a clinical research medical suite.

2.3.1 Visit Standardisation

Dietary Control (All Studies)

Participants were instructed to refrain from alcohol consumption for 48 hr, and avoid caffeine for 12 hr, preceding all visits. Compliance was assessed verbally at each visit, with email reminders sent out three days, and the day, prior. Participants were weighed at the commencement of each visit, to ensure no substantial changes in bodyweight had occurred.

Dietary Control (Chapters 3 & 4)

Participants were asked to consume a high carbohydrate meal >1 hr before Baseline visits in line with best practice for fuelling[3]. Prior to each Experimental visit, participants completed a diet diary for one (*Chapter 3*) or two days (*Chapter 4*). They were asked to use the diary completed before the first Experimental visit as a guide for subsequent visits to reduce the chance of between-visit differences in skeletal muscle/liver glycogen levels[134]. Compliance was assessed by completion of further food diaries. The nature of their diet was not prescribed so that it could reflect personal race preparation habits, but was checked to ensure that they were achieving optimal daily intake of carbohydrate (5-10 g/kg·BW) and protein (>1 g/kg·BW)[3], based on nutritional information obtained from supermarket websites.

Training Load Control

For all studies, participants were instructed to refrain from completing any strenuous training on the day preceding any visits. In *Chapters 3 & 4*, to minimise confounding effects of training-induced fatigue on performance outcomes, participants were requested to keep their training regimes consistent over the course of the study. To facilitate adherence, participants completed a training diary for the six days preceding the first Experimental visit, which they were asked to replicate as closely as possible for the week/s preceding the subsequent visit/s, completing training diaries then also. Experimental visits were spaced a week apart whenever possible to fall at the same point in weekly training cycles.

Menstrual Cycle Control (Chapters 3 & 4)

As neither the influence of menstrual cycle, nor of contraceptives, on endurance exercise performance or metabolism is robustly established[344–346], in line with current recommendations[345] participants were asked about how they perceived their cycle and/or contraceptives to affect their exercise capacities. Unless the participant indicated otherwise, exercise performance and metabolism was assumed to be most stable and predictable in the mid-luteal phase, thus visits were timed to likely fall within this window where possible[346,347]. As steroid hormones levels were not assessed, the luteal phase was assumed to occur 14-28 days after onset of menstruation[345,348], though this predicted window was adapted based on participant feedback.

If a participant started their menstrual cycle, subsequent visits were delayed until six days-post the end of menstruation (self-reported) where they were likely out of the early follicular phase, which is tentatively associated with reduced exercise capacity and alterations in substrate oxidation[345,346].

2.3.2 Screening Call & Baseline Visits

Screening Questionnaire & Informed Consent

For *Chapter 3*, participants provided oral consent for screening at the start of the Screening Call, with a record of this consent sent to participants. For *Chapters 4 & 5*, participants signed a Pre-Screening Consent form at the start of the Baseline visit.

The medical and physical fitness questionnaire used was adapted from the American College of Sports Medicine (ACSM) guidelines[349]. It was completed either during the Screening Call (*Chapter 3*) or at the Baseline visit (*Chapters 4 & 5*). If a participant scored >1 for any risk factors after mitigating circumstances were taken into account, or if they did not match the inclusion criteria, they were not permitted to participate in the study. Participants were then talked through the Participant Information Sheet.

Participants provided written informed consent to enrol into the study at the Baseline visit (*all Chapters*), prior to the start of any testing.

Participants' height (Model 213, Seca, Birmingham, UK) and weight (DP3800/M-100, Marsden Weighing Machine Group Ltd, Rotherham, UK) were also then recorded.

2.3.3 Study Drinks

During all Experimental visits, either ketone monoester (KME; TΔS Ltd, Thame, UK) or placebo drinks were consumed. *Chapters 3 & 4* were double-blinded, and *Chapter 5* was single-blinded, to drink composition.

The KME, providing 4.8 kcal/g, is designated 'Generally Regarded as Safe' by the US Food and Drug Administration (FDA) and does not carry any restrictions under World Anti-Doping Authority (WADA) regulations. Safety, toxicology, and kinetics data are available in published literature[20,28,109,110].

For *Chapter 3*, the placebo consisted of a bitter flavouring (#648352, Symrise, Holzminden, Germany)[65,107,151,168,350]. For *Chapter 4* & 5, the placebo solution consisted of 2 mM Sucrose Octaacetate (#252603, Sigma-Aldrich, MO, USA)[146,157,237,351]. Sucrose Octaacetate is not digested in the body and

therefore carries a negligible caloric value. In all instances, placebo drinks were taste and volume matched to respective KME drinks given at the same timepoint.

All drinks (KME, placebo, and carbohydrate-only) were chilled to 4 °C, provided in opaque sports bottles, and calorie-free concentrated squash flavouring (Mini Orange, Robinsons, Hemel Hempstead, UK) was added at 0.02 ml per 1 ml drink to enhance palatability and aid blinding. Participants were instructed to consume each drink as quickly as possible to ensure timing consistency.

Chapters 3 & 4

KME/placebo drinks were prepared by an independent study investigator who had no direct contact with the participants, to ensure double-blinding.

The composition of pre-/in-exercise carbohydrate drinks, consumed alongside KME/placebo drinks, did not differ between conditions for Experimental visits. Thus dietary supplementation regimes were not isocaloric as a potential mechanism of enhanced performance with the co-ingestion of KME and carbohydrate is that more oxidisable substrate is able to be delivered compared to carbohydrate-alone due to saturation of intestinal simple-sugar uptake[138].

Participants were permitted to drink chilled filtered water during exercise testing. Consumption was *ad libitum* during the first Experimental visit, with the volume recorded and replicated for the subsequent visit/s.

2.3.4 Experimental Visits

Participants arrived at the laboratory approximately 30 min before they would habitually consume breakfast (6.00-9.30 AM) and fasted overnight except for water (>10 hr), with arrival time consistent across all visits ±15 min. Enduring verbal consent was provided by participants at the start of each visit.

For *Chapters 3 & 4,* participants were asked to wear the same exercise clothing (cycling/running/triathlon) for each visit.

Standardised Breakfast

Where provided, the composition of the breakfast was the same across all visits for each participant, such that it provisioned 2 g/kg·BW (bodyweight) of carbohydrate as is best practice for a meal consumed 2 hr prior to exercise[3]. It consisted of porridge (40 g oats, 300 ml semi-skimmed milk, 15 g white granulated sugar [excluded from breakfasts provided in *Chapter 4* due to its high natural abundance[352] of ^{13}C, as a U-^{13}C-glucose tracer was provided; see 4.2.3 for further detail]), white toast with butter & jam, and fresh 'smooth' orange juice (Tesco, Welwyn Garden City, UK), adjusted for allergies where appropriate. Participants consumed the components of the breakfast in the same order (self-determined) across all visits and were encouraged to eat at a similar speed each time.

Any timings relative to the breakfast refer to the point of finishing consumption. Participants were permitted to drink water *ad libitum* after the breakfast until any testing commenced. All exercise testing during *Chapters 3 & 4* was undertaken after participants had consumed this breakfast (i.e. post-prandially) to increase ecological validity.

Blood Sampling

Upon arrival, an antegradely indwelling 20- or 18-gauge cannula (20g: #393224, 18g: #393227, Venflon™ Pro Safety, BD, NJ, USA) was sited in a dorsal vein of the antecubital fossa, with a needle-free valve fitted (#2000E7D, SmartSite™, BD).

At each sampling timepoint, 1 ml of venous blood was drawn and immediately discarded. Samples were then collected into a 4 ml sodium fluoride/potassium oxalate 'Glucose' vacutainer (#368921, BD, UK; *Chapter 3*) or 4.5 ml lithium-heparin syringe aspiration-style tubes (#05.1106.001, S-Monovette®, Sarstedt, Nümbrecht, Germany; *Chapters 4 & 5*) via a multi-adapter (#14.1205, Sarstedt) for plasma, and immediately placed on ice. The cannula was flushed with 0.9% NaCl saline (pH 5.5, 308 mOsm/l; #FKE1323, Baxter Holding B.V., Utrecht, Netherlands) after each draw.

Blood tubes were centrifuged (2000g, 4°C, 10 min; rendered acellular; Centrifuge 5702 R, Eppendorf AG, Hamburg, Germany) immediately after each collection (*Chapters 4 & 5*) or at the end of each visit

(*Chapter 3*). The plasma component was transferred to 0.5 ml tubes (Starlab International GmbH, Hamburg, Germany) to be stored at -30 or -80°C for later analysis.

Subjective Measures: Ratings of Perceived Exertion, Gastrointestinal Distress, & Appetite

Generalised Ratings of Perceived Exertion (RPE; 'What is your overall perceived exertion?', Appendix A) was verbally conveyed by the participant using the 6-20 Borg[353,354] scale.

Localised RPE[285] was also assessed as: *Breathlessness Intensity* ('How breathless are you?'), *Anxiety of Breathing* ('How anxious are you about your breathing?'), *Leg Discomfort* ('What is your leg discomfort?'), and *Anxiety of Leg Discomfort* ('How anxious are you about your leg discomfort?'). These scores were recorded in a randomised order on a linear scale (0, no anxiety/discomfort; 10, maximal anxiety/discomfort; Appendix B). Interpretation of 'anxiety' in each instance was left to the participant's subjective discretion.

Gastrointestinal (GI) Distress was measured using a literature-standard Likert 0-8 scale questionnaire[159,355,356] (Appendix C) quantified at upper abdominal, lower abdominal, and systemic levels (0, minimal; 8, maximal). Scores for each of the 12 symptom measures at a given timepoint were summed to give a 'Total GI Distress' score for each participant.

Appetite sensations were assessed using validated visual-analogue scales[357] (0, minimal; 10, maximal; Appendix D) assessed as: *Hunger* ('How hungry do you feel?'), *Fullness* ('How full do you feel?'), *Satisfied* ('How satisfied do you feel?'), and *How Much Could Eat* ('How much do you think you can eat now?'). A composite score for *Total Appetite* was calculated as the following:

***Equation 2.1* - Total appetite.**

$$Total\ Appetite = (Hunger + How\ Much\ Could\ Eat) - (Fullness + Satisfied)$$

Questions assessing all subjective scores were asked by the same investigator throughout, who was condition-blinded in *Chapters 3 & 4*. Participants were fully re-/familiarised to the scales at each visit.

2.3.5 Cycle Ergometer

In *Chapters 3 & 4*, participants undertook all cycling exercise on a magnetically braked ergometer (Ergoselect 4, Ergoline GmbH, Bitz, Germany; calibrated annually), in a set-power cadence-independent mode. Power requirements were controlled via Metasoft Studio software (Metasoft Studio v5.12.0, Cortex Biophysik GmbH). Participants could monitor their cadence on a digital display.

Participants used their own clipless pedal/cleat systems where possible, otherwise pedals were provided, as well as their own cycling shoes and cycling-specific clothing. The saddle and handlebars were set to their preferred position during Baseline visits and replicated across all subsequent visits.

2.3.6 Cardiorespiratory Measures

Respiratory Gas Exchange

For periods of respiratory gas exchange analysis (also referred to as 'indirect calorimetry'), participants wore an appropriately sized well-sealed face mask (Hans Rudolph, USA) and breathed through a low-resistance, single-use, pre-calibrated turbine (236-01-020, Cortex Biophysik GmbH, Leipzig, Germany), with breath-by-breath gas composition and volume monitored continuously using an online analysis system (Metalyser 3BR3, Firmware v1.04.10, Cortex Biophysik GmbH). A two-point calibration protocol was undertaken before each testing session in line with the manufacturer's instructions, as 1. ambient air, and 2. 4.99% CO_2 & 15.04% O_2 gas (Cranlea, Birmingham, UK). Barometric pressure and temperature were determined automatically by the system. Cooling fans were shut off during gas analysis periods in case they influenced turbine airflow.

Cardiorespiratory data collection periods were visualised in real-time via the Metasoft Studio software, though participants were always blinded to this data. Raw data was exported to Excel (Microsoft Corporation, WA, USA) for further analysis with clearly aberrant values (those that were physiologically impossible) omitted. The following datasets were collated to be reported/for further analyses: rate of oxygen consumption ($\dot{V}O_2$), rate of carbon dioxide production ($\dot{V}CO_2$), respiratory exchange ratio (RER; $\dot{V}CO_2/\dot{V}O_2$), tidal volume (V_T), breathing frequency (BF), and minute ventilation (V_E).

The first minute of any respiratory gas sampling period was excluded from analyses in case the act of fitting the mask caused any transient disturbance to breathing patterns to allow for a steady state to be (re-)established[253]. The first minute was also excluded after any power change in *Chapter 4*. Data were then averaged across the remaining period.

In *Chapters 3 & 4*, heart rate was quantified continuously across the gaseous exchange sampling time periods via a chest-strap monitor (H10, Polar® Electro, Espoo, Finland) connected to the Metalyser system by Bluetooth.

Criteria for Establishment of Ventilatory Threshold 2 and $\dot{V}O_{2peak}$ (Baseline visits, Chapters 3 & 4)

Ventilatory Threshold 2 (VT2) was initially automatically calculated in the Metasoft Studio software. Data was then checked visually for a second V_E-$\dot{V}O_2$ breakpoint, confirmed by a deviation from stability in $V_E/\dot{V}CO_2$-$\dot{V}O_2$[358,359]. $\dot{V}O_{2peak}$ was determined as a plateau in $\dot{V}O_2$, taken as the highest rolling 30 sec average with deviations of <5% from that average. Alongside this, at least two of the following criteria needed to have been met: RPE at volitional exhaustion (Borg 6-20 scale[353,354]) ≥18, heart rate within 10 bpm of self-reported maximum, RER >1.10 for >30 sec. The test was also considered complete if their cadence fell >15 rpm below their average cadence up until that point, or under 60 rpm, for >10 sec, despite strong verbal encouragement. There was an assumed lag of 20 sec between oxygen unloading into exercising muscle and arrival into pulmonary gas exchange vasculature[360].

Substrate Oxidation Calculations for Experimental Visits (Chapters 3 & 4)

The presence of KBs as oxidisable substrates in addition to carbohydrate, fat, and protein confounds the assumptions necessary for traditional interpretation of RER to calculate substrate oxidation rates[69]. Data on KB oxidation rates under exogenous ketosis is too sparse to confidently extrapolate from[70,71], especially in a postprandial state where no βHB stable isotope tracer-labelled studies have been conducted. Tracer work in a fasted state indicates that previous estimates of βHB oxidation during exercise, calculated from disposal rates[107], were ~two-fold overestimates[70,71], thus rate-of-disappearance methodologies cannot be confidently used here. Additionally, gluconeogenesis with alanine as the

substrate involves fixation of CO_2 into carbamoyl-phosphate, a process requiring energy and one that may influence RER values[257] (RQ: 0.13, assuming energy derived from palmitate oxidation[69]). Up to 25% of glucose production was determined to result from gluconeogenesis during 90 min cycling at 65% $\dot{V}O_{2max}$[361], and up to 45% during 4 hr fasted ~30% $\dot{V}O_{2max}$ cycling[362], with gluconeogenesis from alanine increasing across 2 hr of 40% $\dot{V}O_{2max}$ cycling. Thus alanine-derived gluconeogenesis likely tangibly contributes to endogenous carbohydrate provision and energy expenditure during the latter stages of the testing protocols in both *Chapters 3 & 4*. As exogenous ketosis is known to supress rates of gluconeogenesis, in part through reducing circulating alanine avaliability[107,136], one cannot assume that gluconeogenesis rates, and its influence on RER (and thus substrate oxidation calculations), are equivalent between KME and placebo conditions. As indirect calorimetry data could not be confidently corrected for ketone oxidation nor ketosis' indirect effects on substrate synthesis, substrate oxidation rates were not calculable under any exogenous ketotic states.

Protein oxidation during exercise was assumed to be negligible. Even in extreme settings, the contribution of protein to energy expenditure (EE) is unlikely to exceed 10%[197,257,363–365], and would likely be below that in this work as exogenous carbohydrate was provisioned throughout and exercise commenced with replete glycogen stores. Hepatic lipogenesis was also taken as negligible due to the caloric-deficit nature of the exercise supressing this process[257,366,367] (though this assumption was challenged by findings in *Chapter 3*), whilst adipose lipogenesis was assumed to be trivial in the trained athlete population studied[368]. Any ketone bodies (KB) endogenously produced in the placebo conditions (ketogenesis would be supressed under KME conditions[38]) and subsequently oxidised are already encompassed within fat oxidation calculations[69,257].

Substrate oxidation rates were also not calculated across the 10 km run (*Chapter 3*) or time-to-exhaustion trial (*Chapter 4*) as a metabolic steady state could not be assumed[142,253].

2.4 Analytical Procedures

All samples for a given participant had been frozen at the same temperature and had undergone the same number of freeze-thaw cycles (kept to a minimum, always ≤2), were extracted/isolated at the same time, and analysed at the same time/in the same run/on the same ELISA/Multiplex plate, as appropriate.

Plasma & Urine Biochemistry Analyses

All plasma & urine samples were biochemically assessed using a clinical chemistry analyser (Pentra C400, Horiba Medical, France [*Chapter 3*]; AU480, Beckman Coulter, CA, USA [*Chapters 4 & 5*]). Reagent preparation, calibration, and quality control procedures were all in-line with manufacturers' recommendations (*Table 2.1*). Additionally, a plasma sample with known metabolite concentrations was analysed alongside any study samples as validation of run-to-run consistency. Samples were diluted 9:1 with 0.9% saline to fall within detection range if required. All *Chapter 3* samples were analysed in duplicate. For *Chapters 4 & 5*, any samples with aberrant findings (physiologically impossible/calculated outliers) were re-analysed twice with the three values averaged if they agreed, otherwise the two values most in agreement were averaged. Non-HDL-cholesterol was calculated by deducting HDL-cholesterol from total cholesterol.

***Table 2.1* - Plasma Biochemistry Reagents**

Analyte	Product Code	Manufacturer
Glucose	0018250840	quantiLAB, Milano, Italy
TAG	0018255640	quantiLAB, Milano, Italy
βHB ('Ranbut')	RB1008	Randox Laboratories, Donegal, Ireland
NEFA	FA115	Randox Laboratories, Donegal, Ireland
Lactate	LC2389	Randox Laboratories, Donegal, Ireland
Glycerol	GY105	Randox Laboratories, Donegal, Ireland
Urea	UR8334	Randox Laboratories, Donegal, Ireland
Total Cholesterol	0018250540	quantiLAB, Milano, Italy
HDL-Cholesterol	CH2655	Randox Laboratories, Donegal, Ireland
ApoB	LP3839	Randox Laboratories, Donegal, Ireland

ApoB, Apolipoprotein B100; βHB, beta hydroxybutyrate; HDL, high density lipoprotein; NEFA, non-esterified fatty acid; TAG, triacylglycerol.

βHB Disposal Estimates (Chapters 4 & 5)

Total βHB disposal rates during KME visits were estimated. Firstly, total blood volume (TBV) for each participant was calculated using the Allen formula, considered the most valid predictor for healthy individuals[369–371]:

***Equation 2.2* - Total blood volume.**

$$Total\ Blood\ Volume\ (L) = (a \times H^3 + b \times W + c)/1000$$

H, height (cm); W, weight (kg). For males: a = 0.000417 mL/cm^3; b = 45.0 mL/kg; c = -30 mL. For females: a = 0.000414 mL/cm^3; b = 32.8 mL/kg; c = -30 mL.

The total molar amount of βHB in circulation at the end of the 4 hr protocol (*Chapter 4)* or at 6 hr (*Chapter 5*) was calculated by multiplying the TBV by [βHB]. The total molar amount of βHB (104 g/mol) provisioned was calculated as twice the molar amount of KME provided (176 g/mol) as one molecule of KME delivers two molecules of βHB into circulation[20,110,214]. It was assumed that all of the KME had been hydrolysed and taken up into circulation in the GI tract across the assessed time period[23] and that none was lost into faeces. Estimates were not made for *Chapter 3* as urine was not collected across Experimental visits, it could not be assumed that all βHB had been taken up into circulation during the 1 hr cycle, and no data exists for βHB oxidation during running[70,71].

Total disposal was calculated as the amount of KME provided, minus the total amount in circulation at 4 hr, accounting for the amount lost in urine (for participants without urine data in *Chapter 4*, the average from participants with data, 2.197 mmol, was used). βHB oxidation was assumed to average 6.00 g/hr (*Chapter 4*) and 0.54 g/hr (*Chapter 5*) based on comparable data established from stable isotope methodology[71]. AcAc was assumed at 0.6 mM AcAc, taken from studies with the closest comparable settings to work here[23,107], and alternatively at 2.0 mM, the highest value reported in the literature from KME ingestion[125].

2.5 Data Analysis

Data are presented as mean ± SEM unless otherwise stated. Significance was taken at $p < 0.05$. A trend for significance was taken at $0.05 \leq p < 0.10$, with $0.07 \leq p < 0.10$ considered a 'weak' trend. Outliers were identified using the ROUT method[372] (Q = 1%), their inclusion/exclusion was assessed on a case-by-case basis.

Normality assumptions for each timepoint under each condition (ANOVA/correlations/regressions) or residuals (t-test) were assessed with Shapiro-Wilk[373] tests. Non-normally distributed data were transformed in the following ways depending on their nature:

- Moderately positively/negatively skewed: 'square-root'/'reflect & square-root', respectively
- Strongly positively/negatively skewed: '$\log_{10}$'/'reflect & $\log_{10}$', respectively

Reflected data had all values subtracted from the 'largest value in the dataset plus one unit', before having the transformation applied. If a condition/timepoint was transformed, all data for that given measure were transformed in the same way with statistical analyses performed on the transformed data set, though original data are presented graphically and in tables for ease of interpretation.

Where Pearson's correlations or simple linear regressions[374] were performed, linearity of data points (as homoscedasticity and normality of residuals) was confirmed via visual inspection.

The effect of condition on measures with multiple timepoints was assessed using two-way repeated-measures ANOVAs[375]. A one-way repeated-measures ANOVA was used to assess substrate oxidation and energy expenditure over time in placebo conditions-only and for any single timepoint measures/AUCs in *Chapter 3* (3 conditions). The effect of condition on any single timepoint measures/AUCs in *Chapters 4 & 5* (2 conditions) was assessed using paired t-tests (two-tail). Mauchly's Test[376] was used to ascertain sphericity where ≥1 factor had ≥3 levels. If the assumption of sphericity was not met ($p < 0.05$ *and/or* Greenhouse-Geisser $\varepsilon > 0.75$) then a Greenhouse-Geisser correction[377] was applied. Baseline (pre-intervention) differences were assessed using a paired t-test or one-way ANOVA, as appropriate, in case they needed to be applied as a co-variate for ANCOVA analysis, though this was not necessary for any data presented in this thesis.

Post-hoc Šídák multiple comparisons tests[378] were undertaken to explore between-condition differences when justified by a significant condition-by-time interaction effect, or condition effects where ≥3 conditions were present. In some specific instances exploratory post-hoc analyses were undertaken with a trend for an interaction effect/without a corresponding interaction effect (rationale detailed with results). Post-hoc multiple comparisons for time within a given condition were not performed to retain statistical power for between-condition comparisons[378], unless otherwise stated.

For *Chapters 3 & 4*, which were double-blinded, data collation and statistical analyses for delta economy, gross economy, time-to-exhaustion/time trial time, subjective measures, and cardiorespiratory measures (as applicable) was undertaken still blinded, in that order, with biochemistry analyses always performed last.

Area-Under-the-Curve

Area-under-the-curve[379] (AUC) was calculated using the trapezoid method with y = 0 as the baseline unless otherwise stated. Incremental AUC (iAUC) was not used as at least one datapoint fell below baseline values for the majority of datasets analysed. AUCs were assessed across the timepoints presented graphically, or in a table, for the given metric.

Software

Power calculations were performed in G*Power v3.1.9.6[380] (Heinrich-Heine-Universität Düsseldorf, Germany). Data were collated in Excel (Microsoft Corporation). All statistical analyses were performed in SPSS Statistics© v30.0.0.0 (IBM, Chicago, IL, USA). All figures were generated in GraphPad Prism™ v10.4.2 (GraphPad Software Inc, San Diego, CA, USA).

Chapter 3 - The Dose-Dependent Influence of Ketone Monoester Ingestion on Multimodal Endurance Performance in a High-Carbohydrate Postprandial State

3.1 Introduction

Evidence for exogenous ketones enhancing exercise performance is equivocal with only a minority of studies reporting improvements[31,32,381]. The discrepancy between findings may be in part explained by heterogeneity in:

i. the provision of other macronutrients, both pre- and in-exercise, and

ii. the degree of ketosis achieved[31].

Whether participants undertook testing in a postprandial (fed) or postabsorptive (fasted) state presents a major source of variability across studies exploring the KME and exercise. Seminal work by Cox et al.[107], who found a 2% benefit to 30 min cycling time trial (TT) performance under exogenous ketosis (2-2.5 mM βHB; 'study 5'[107]) after 1 hr at 75% W_{max}, had participants arrive after an overnight fast with no breakfast provided. Whilst in-exercise carbohydrate was delivered at ~40 g/hr, oxidation rates likely far exceeded this exogenous provision given the high power outputs (male: 303 W, female: 212W at 75% W_{max}; 8 male/2 female) of the athletes working at almost entirely carbohydrate-reliant intensities (RER 0.98-1.05)[3,5,265,382]. Thus, glycogen stores would not be repleted and a functionally fasted state would have been maintained throughout, which carries limited real world translatability[3,383]. The majority of subsequent studies exploring the impact of exogenous ketosis on acute exercise performance have used a variant of Cox et al.[107]'s 'submaximal preload exercise bout followed by a performance test'

protocol[94,125,146,150,154,230,232,233,241]. Despite this, only two have established a positive endurance performance outcome (as opposed to a determinant/predictor of performance[71,153,232]) with participants in a postprandial state[157]. One concerned rugby match simulation with reaction time as a component of the testing[231], so its applicability to wider endurance sport is restricted. The positive finding in the other required co-ingestion of sodium bicarbonate with the KME however, with no effect seen in the KME-only arm, and βHB levels were only elevated (~2-3 mM) across 1-2 hr of a 3 hr 'intermittent' cycling preload and not during the subsequent 15 min TT (<0.5 mM)[157]. Further obfuscating the interaction between exogenous ketosis and postprandial metabolism, pre-exercise feeding strategies vary considerably across this literature. Hence it remains equivocal what the influence of exogenous ketosis might be on endurance exercise performance proceeding a 'gold standard'[3] pre-exercise meal, where KB levels are consistently elevated throughout the testing protocol.

Similar to the variability in feeding state, the dosages of KME used, thus the circulating βHB levels achieved, are inconsistent between studies. It is therefore uncertain whether an 'optimal' range for [βHB] exists with regards to exercise performance. Whilst saturation for KB uptake into skeletal muscle (SM) appears to occur at 1-2 mM[53,72], this threshold was established at rest in healthy but non-exercise trained participants. Type I muscle fibres appear to carry the greatest KB uptake[74] and oxidative[70,73,75] capacities, with performance-oriented endurance athletes likely to be more type I dominant than the general population given the competitive benefits associated with this phenotype[81–83]. Furthermore, endurance training potentially increases ketolytic capacity two-to-threefold[76], though human studies are lacking, whilst exercise itself may raise the ceiling for KB oxidation with disposal rates ~5x those seen at rest[384,385]. Thus this 1-2 mM ketone uptake saturation range may not apply to an exercising trained endurance athlete model. Limited human work employing ^{13}C-labelled KME has, though, observed that βHB oxidation was only 5.1% greater at circulating levels of 4.33 mM compared to at 1.87 mM during fasted 75% W_{max} cycling in trained athletes (0.103 vs 0.098 g/min)[71], suggesting utilisation might start to plateau around 2 mM during exercise in this population. Accordingly, it has been proposed that the 'optimal' ergogenic range for ketosis in exercise might be ~1-3 mM[24,32]. This is based on the rationale that below these bounds KB availability for oxidation might be insufficient to drive tangible shifts in metabolism, whilst above them no further increase to oxidation rates may be seen but potentially

deleterious characteristics of exogenous ketosis, such as ketoacidosis[152,157] and gastrointestinal disturbance[20,159], likely become increasingly severe.

This optimal range concept tentatively aligns with existing performance research. For example, an ergogenic benefit of the KME was seen by Cox et al.[107] at 2-2.5 mM plasma βHB but not in a similarly structured treadmill-running protocol (1 hr at 65% $\dot{V}O_{2max}$ then a 10 km TT) where βHB peaked at 1.13-1.52 mM[154]. This might indicate that the lower-threshold for ketosis sits at ~1.5 mM, though this comparison is confounded by differing feeding states. Nonetheless, more widely, studies utilising exogenous ketone supplements (EKS) that do not raise levels above 1.5 mM (1,3-butanediol, MCTs/MCFAs, and ketone salts) have failed to observe performance benefits[31,33,34]. Furthermore, during a 30 min cycling TT where plasma βHB levels were on average 3.5 mM, higher [βHB] correlated with greater impairment to performance when assessed at an individual level[152], supporting the existence of an upper-threshold. Whether there exists a dose-response relationship between circulating βHB and exercise performance, and if this agrees with the proposed 1-3 mM range, is yet to be directly interrogated, however.

The aim of this study was therefore to investigate whether exogenous ketosis exhibits a KME dose-dependent enhancement in endurance exercise performance, in an ecologically valid postprandial high-carbohydrate state. The exercise model employed here was a combination of cycling and running as, although interest surrounding the KME in du/triathlon is high[30,228], no exogenous ketosis study has yet focussed on multimodal performance.

3.2 Methods

COVID-19 Impact

As data collection for the study took place in 2021-22, all steps necessary to minimise the risk of COVID-19 transmission were taken. These measures were in-line with departmental, university, and governmental regulations in place at a given time and a supplementary COVID-19 information sheet was sent to participants before they were enrolled outlining what would be asked of them and the precautionary measures in place. The start of the study was still, however, delayed by several months due to departmental regulations prohibiting human research. Recruitment was subsequently hindered throughout, with a number of potential participants citing concerns surrounding government regulations (isolation requirements etc) and risk of infection as the reason for not proceeding to enrol in the study. This is reflected in the high attrition rate seen between the Screening Call and Baseline visit. Additionally, two participants did not complete the final Experimental visit due to contracting COVID-19 (*Figure 3.1*).

3.2.1 Study Design

This was a double-blind, randomised, and counterbalanced, repeated measures design study. Participants completed a screening video-call, then a Baseline testing and protocol familiarisation visit, followed by three Experimental testing visits (*Figure 3.2*).

During the Baseline visit, participants' cycling $\dot{V}O_{2peak}$ was established, and they undertook a familiarisation run on the treadmill.

During the Experimental visits, participants consumed drinks containing a placebo (PLA), a low dose of KME (Low-KME), or a high dose of the KME (High-KME) alongside carbohydrate (CHO) drinks. Under each of these PLA/Low-KME/High-KME conditions, participants completed 1 hr steady state cycling at a power output (PO) corresponding to 70% $\dot{V}O_{2peak}$ (cycle leg) before transitioning to a treadmill to run a 10 km TT (run leg). A randomly generated balanced matrix was used to ensure each dose-condition order was equally represented across the study to prevent order-effect biases, with participants assigned a predetermined visit order based on enrolment date.

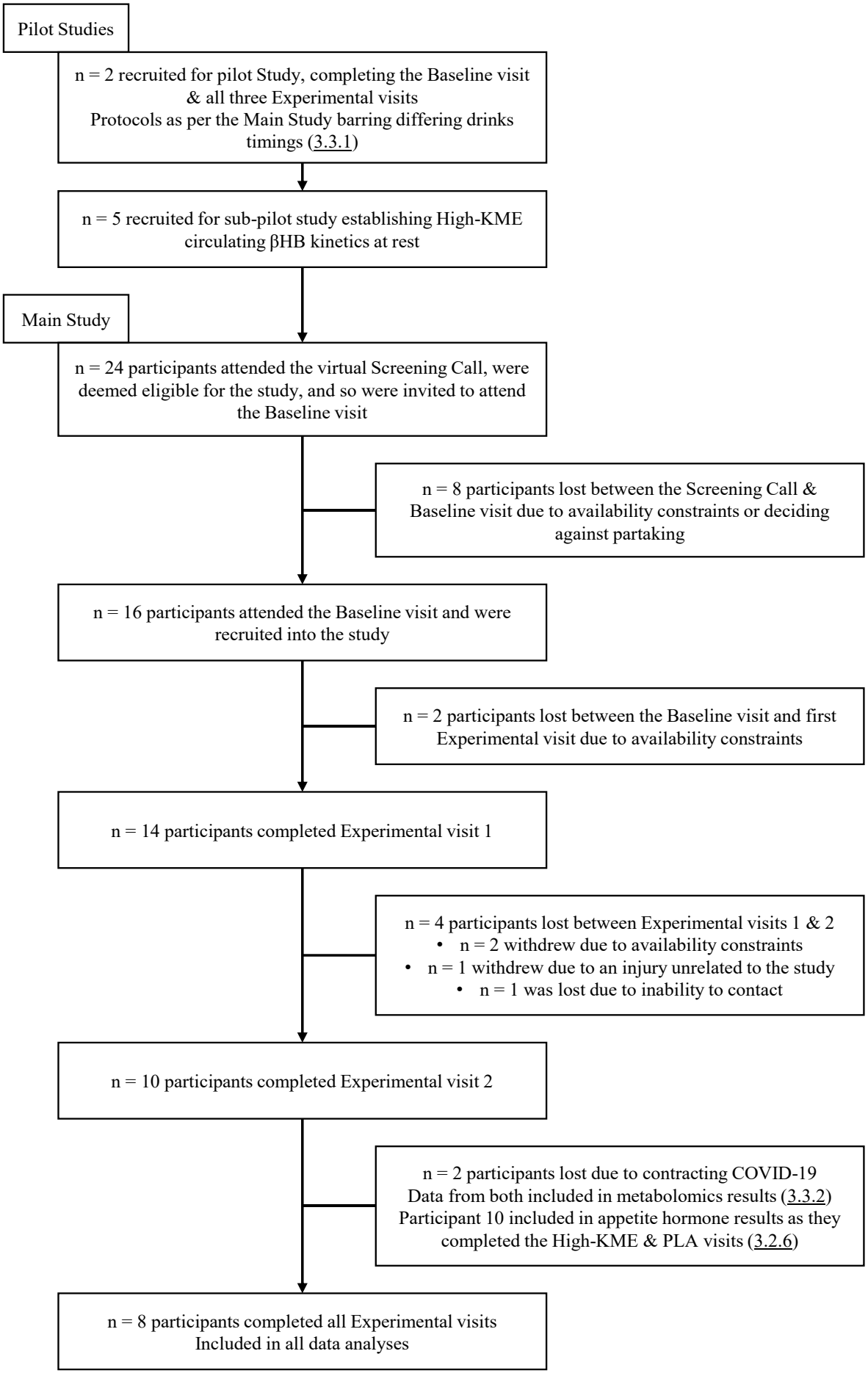


***Figure 3.1* - Study CONSORT diagram.**

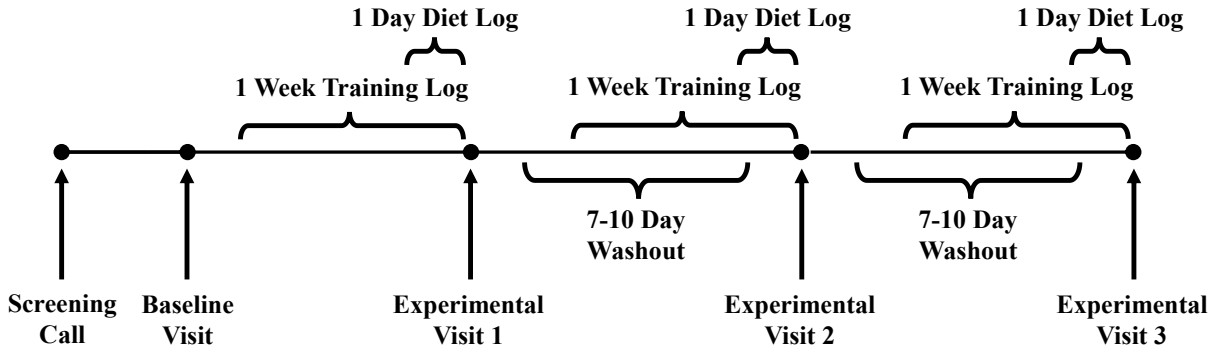


***Figure 3.2* - Overview of study timeline.**
Baseline and first Experimental visit separated by at least four days.

Participants

Eight participants were recruited from a pool of local multisport athletes in the Oxfordshire & Greater London area. A further two participants were recruited and completed only two of the three Experimental visits (withdrawing due to contracting COVID-19), with their results included in metabolomics and plasma hormone datasets only (*Figure 3.1*). Inclusion criteria are found in 2.2. Participant characteristics are summarised in *Table 3.1* (n = 8) and *Table 3.2* (n = 2).

***Table 3.1* - Participant characteristics, n = 8 who completed all three Experimental visits.**

		Male			*Female*		
n			6			2	
Age	yr	25.8	±	2.3	22.0	±	0.0
Height	cm	179.9	±	2.1	162.6	±	2.0
Weight	kg	71.6	±	2.7	59.1	±	1.7
70% $\dot{V}O_{2peak}$	W	263.2	±	8.7	166.5	±	3.2
VT2	W	303.7	±	15.9	211.5	±	0.9
	ml/min/kg	53.8	±	1.9	46.5	±	1.4
$\dot{V}O_{2peak}$	ml/min/kg	64.0	±	2.0	53.0	±	3.5

BMI, Body Mass Index; VT2, Ventilatory Threshold 2. Data presented as Mean ± SEM.

***Table 3.2** - Participant characteristics, n = 2 who completed two of three Experimental visits.*

Participant		*9*	*10*
Gender		M	F
Age	yr	21	28
Height	cm	182	166
Weight	kg	69.9	62.6
70% $\dot{V}O_{2peak}$	W	326	233
VT2	W	330	269
	ml/min/kg	58	51
$\dot{V}O_{2peak}$	ml/min/kg	78	58

BMI, Body Mass Index; VT2, Ventilatory Threshold 2. Results from these participants is included in plasma metabolomics and plasma hormone datasets only. Data presented as Mean ± SEM.

3.2.2 Pilot Work

A n = 2 pilot was conducted prior to commencement of the main study to refine the methodology, focussed primarily on:

1. Ensuring that the prescribed intensity for the cycle leg was appropriate, i.e. taxing but sustainable for the full hour,
2. Optimising KME drink timings & dosages to provide consistent plasma βHB concentrations across the cycle leg, establishing a clear difference between High-KME and Low-KME conditions, and
3. To ensure the High-KME dosage could be tolerated.

This was a full-protocol pilot, i.e. Baseline and three Experimental visits, with methodology the same as the main study except for the consumption of Drink 1 (*Table 3.5*) being at -40 min rather than -60 min as in the main study, a change made due to findings from this, and the below, pilot work (3.3.1).

This was followed by a n = 5 sub-pilot focussed only on resting circulating βHB kinetics. Each visit replicated an Experimental Visit from arrival through until 150-160 min after the drink had been consumed, with drinks given matching those of Drink 1 (both KME and carbohydrate) during a High-KME visit. Capillary blood [βHB] (Freestyle Optium Neo H, Abbott Laboratories, Chicago, IL, USA) was measured fasted, at the point of Drink 1 ingestion, and across the proceeding period. These drinks

were consumed either 1 hr ('-60 min', as in the main study) or 1 hr 20 min ('-40 min', as in the n = 2 pilot) after the standardised breakfast had been finished.

3.2.3 Screening Call & Baseline Visit

During an eligibility video call, participants were screened as per 2.3.2, then gave verbal consent to being enrolled into the study.

Participants subsequently attended the in-person Baseline visit where they provided written informed consent (2.3.2), then completed a cycle step-test and a familiarisation treadmill run. Cardiorespiratory measures were monitored continuously in both (2.3.6).

To establish $\dot{V}O_{2peak}$ (criteria: 2.3.6), participants undertook a self-paced warm-up for 10 min before immediately transitioning to a step test (30 W increase every 2 min) starting at 180 W (male)/90 W (female) until volitional exhaustion.

The PO of the Experimental trials' cycle leg was determined using a regression equation which plotted $\dot{V}O_2$ for the final 30 sec of each step versus power, with 70% of the $\dot{V}O_{2peak}$ value (in mL/min/kg) used to find the respective PO.

Following 10 min active cool down on the cycle ergometer, participants transitioned to the treadmill (4Front, Woodway, Wisconsin, USA) and undertook a 10 km familiarisation run at 'race pace', acting to minimise any learning effects[386]. Completion of this 10 km informed the Experimental visits' run leg starting speed, though participants were not told what this would be. The respiratory gas exchange face mask was worn throughout to diminish the novelty of this setup, and the cycle-to-run transition of the Experimental visits was practiced as this involved removal and re-fitting of said mask.

3.2.4 Experimental Visit Standardisation

Training Load & Dietary Control

Participants kept training load consistent for the six days prior to each visit and standardised their diet for the day preceding each Experimental visit, both as described in 2.3.1.

Menstrual Cycle Control

Two of the three female participants were not on any form of contraceptive, whilst one had a progestogen intrauterine device fitted. Details on how visit timings were controlled for menstrual cycle are described in 2.3.1.

3.2.5 Experimental Visits

Participants undertook three Experimental testing visits, with the protocols only differing in whether they consumed High-KME, Low-KME, or PLA drinks. The protocol is described in *Figure 3.3*.

	Rest				*Cycle*					*Run*						
	FASTED	-2 hr	-60 min*	-10 min	0 min	15 min	30 min	45 min	60 min	0 km	2 km	4 km	5 km#	6 km	8 km	10 km
Blood	X	*Std. Breakfast*	X	X	X		X		X				X			X
Indirect Calorimetry				X		X	X	X	X	*Throughout*						
General RPE Scale					X		X		X	X	X	X		X	X	X
Localised RPE Scales					X		X		X		X					X
Appetite Scales	X		X		X											X
GI Scales					X		X		X				X			X

***Figure 3.3* - Experimental testing visit sampling timeline.**

*X, measure collected at that timepoint. *, first KME/PLA drink (Drink 1) consumed immediately after the -60 min timepoint. #, 10 km run paused at 5 km for 120 sec to obtain blood sample. Indirect calorimetry (respiratory gas exchange) data collected across the 4 min prior to the respective timepoint during Rest and Cycle. GI, Gastrointestinal; RPE, Rating of Perceived Exertion; Std., Standardised.*

Upon arrival, participants were cannulated (2.3.4) and fasted blood samples obtained. Participants consumed a standardised breakfast (*Table 3.3*) then sat at rest for 1 hr 50 min, during the final 4 min of which indirect calorimetry respiratory gas sampling was undertaken.

At 1 hr 50 min post-breakfast they commenced a 10 min cycling warm-up (5 min at 100 W, then 5 min at 50% $\dot{V}O_{2peak}$ power) before immediately transitioning into the 1 hr cycle leg, set at a PO corresponding to 70% of their $\dot{V}O_{2peak}$. Prior to, and during, this cycle leg, KME/PLA and CHO drinks were consumed (detailed below).

Following completion of the cycle leg, participants had 120 sec to change into their running shoes and position themselves on the treadmill with the gas sampling mask refitted.

The run leg (10 km TT) then began with the treadmill speed controlled by a condition-blinded investigator, taking instruction from the participant when they wanted the speed changed. The initial speed was set at a pace deemed suitable from the Baseline visit and was the same across all Experimental visits. Participants were instructed to give their 'best effort' for the full 10 km distance and to pace the effort as they would a race. Participants were blinded to all metrics except for distance covered, which was provided verbally by the researcher at 1 km intervals, plus every 100 m during the 5th and 10th kilometres, and on request. The treadmill was paused at 5 km for 120 sec (deducted from the final time) for blood to be drawn, with participants immediately stepping off the running-belt. The 10 km blood sample was taken immediately upon TT completion. Four of the participants wore carbon-fibre plated 'super shoes'[387,388] for the 10 km run. There appears to be no interactive relationship between exogenous ketosis and these shoes[233].

Participants were not permitted to listen to music in case this might influence their perceived exertion and so that they could hear the study investigator.

Standardised Breakfast

A standardised breakfast was consumed 2 hr before commencing the cycle leg, as per 2.3.4 (exact macronutrients: *Table 3.3*).

***Table 3.3* - Standardised breakfast macronutrient compositions.**

Total Calories	kcal	904	±	45
Carbohydrate	g	138.6	±	5.2
Protein	g	23.5	±	0.8
Fat	g	26.6	±	2.8

Data presented as Mean ± SEM.

Hydration Status

Hydration status, as urine specific gravity (USG), was evaluated when the participant first urinated (Multistix® Reagent Strips, Bayer, Berlin, Germany). To ensure safety and consistency across visits, if a participant was found to be hypohydrated (USG > 1.020), they were instructed to consume further water and were reassessed when they next urinated. There were no instances of hypohydration by the point of exercise commencing.

Carbohydrate & KME/Placebo Drinks

Participants consumed carbohydrate drinks 1 hr after finishing their breakfast (Drink 1; 8.3% solution) and across the cycle leg (Drinks 2-5; 7.5%), see *Table 3.4*. The carbohydrate regime was identical for all three Experimental visits. 75 g/hr in a 2:1 glucose-to-fructose ratio was chosen to replicate/exceed best-practice carbohydrate fuelling for exercise lasting 1-2 hr[3,5,144,243].

No carbohydrate drink was given at 60 min as this would have slowed the cycle-to-run transition, which involved the removal and re-fitting of the respiratory gas exchange face mask. No carbohydrate was provided during the run as the face mask was fitted throughout, and any consumed at the 5 km 120 sec pause would not be absorbed and oxidised in the ~15-20 min remaining to any consequential extent[389,390]. Participants were, however, allowed to drink up to 300 ml plain water at this 5 km pause for thermal comfort. The volume consumed during the first Experimental visit was recorded and replicated for subsequent visits.

***Table 3.4* - Timing and composition of carbohydrate supplemental drinks.**

Drink	Ingestion Time (min)	Water (mL)	Glucose (g)	Maltodextrin (g)	Fructose (g)
1	-60	300	8.33	16.66	0
2	0	250	4.16	8.33	6.25
3	15	250	4.16	8.33	6.25
4	30	250	4.16	8.33	6.25
5	45	250	4.16	8.33	6.25

Timings relative to the start of the cycle leg.

Additionally, participants consumed KME and/or PLA with Drinks 1, 2, and 4 (*Table 3.5*), with doses scaled relative to body-weight (BW). This equated to total absolute amounts of KME given as 59.57 ± 2.25 g (mean ± SEM) for High-KME, and 39.71 ± 1.50 g for Low-KME, for the n = 8 participants who completed all visits (average weight, 69.3 ± 2.6 kg). This regime was chosen to ensure plasma βHB rose to a >2 mM plateau for High-KME and ~1 mM for Low-KME[23,24,107,110], with the subsequent drinks maintaining this elevation. The PLA solution was prepared and provided to participants as per 2.3.3.

***Table 3.5* - Timing and composition of KME and PLA drinks.**

Drink	Ingestion Time (min)	High-KME		Low-KME		Placebo	
		KME (mg/kg·BW)	PLA (mL/kg·BW)	KME (mg/kg·BW)	PLA (mL/kg·BW)	KME (mg/kg·BW)	PLA (mL/kg·BW)
1	-60	573	0	286.5	286.5	0	573
2	0	143.25	0	143.25	0	0	143.25
4	30	143.25	0	143.25	0	0	143.25
Total KME (mg/kg·BW)		*859.5*		*573*		*0*	

Timings are relative to the start of the cycle leg. KME, ketone monoester; PLA, placebo. KME/PLA drink compositions scaled to bodyweight in kg (BW). Low-KME and PLA drinks were taste and volume matched to the respective High-KME drink.

3.2.6 Blood Sampling

Eight venous blood samples were collected during each Experimental visit (*Figure 3.3*) as described in General Methods 2.3.4. Per 2.4, plasma beta-hydroxybutyrate (βHB), glucose, non-esterified fatty acid (NEFA), and lactate concentrations were analysed at all sampling timepoints.

Drink 1 (first KME/PLA drink) was given after the -60 min blood sample was taken and thus -60 min acts as the pre-intervention baseline. Fasted samples were analysed separately to confirm participants had arrived at each visit in comparable metabolic states.

Fasted, postprandial resting (-10 min), and postprandial in-exercise (+60 min) plasma samples from all conditions were analysed using a high throughput NMR metabolomics platform (Nightingale Health, Helsinki, Finland). -10 and +60 min time points were under a state of ketosis where applicable as the first KME/PLA drink was consumed at -60 min, with Fasted acting as the baseline.

The following plasma appetite/satiety-related hormone assays were independently undertaken by Alexander Wythe as part of his DPhil research. The raw data has been provided for analysis here with his permission.

The following measures were assessed in the High-KME and PLA conditions only.

Plasma growth differentiation factor-15 (GDF-15) was assessed using an ELISA (Invitrogen Human GDF15 ELISA Kit #BMS2258, Thermo-Fisher Scientific, MA, USA) according to the manufacturer's instructions and read using a CLARIOStar Plus Microplate Reader (BMG Labtech, Aylesbury, UK).

Plasma levels of insulin, glucagon, total glucagon-like peptide-1 (GLP-1), total ghrelin, pancreatic polypeptide (PP), total peptide YY (PYY), and leptin were quantified using a multiplex assay (U-PLEX Human Microbiome Combo 1 #3-2000-02D286-A, MSD, Rockville, MD, USA) according to the manufacturer's instructions. Samples were diluted 1:3 with Metabolic Assay Working Solution and Blocker D-R (#R93BR, MSD) was added where specified. The plate was read on a MESO QuickPlex SQ120 reader (MSD).

3.2.7 Cardiorespiratory Measures

Respiratory gas exchange (indirect calorimetry) data was collected as described in 2.3.6 for 5 min prior to the warm-up, four five-minute blocks during the cycle leg (10-15/25-30/40-45/55-60 min), and continuously across the two 5 km segments of the 10 km run (*Figure 3.3*).

Gross Economy

Gross economy was quantified during the cycle leg as the average $\dot{V}O_2$ divided by the set-power:

***Equation 3.1* - Gross economy.**

$$\frac{\dot{V}O_2\ (L/min)}{\text{Work Rate (W)}} \quad \text{(Units: L/min/W)}$$

Substrate Oxidation

Total carbohydrate (CHO) and fat oxidation rates (g/hr) alongside energy expenditure (EE; kcal/hr) were calculated from $\dot{V}O_2$ and $\dot{V}CO_2$ (both l/min) for the PLA visits. At rest the equations proposed by Frayn[69] were utilised. During the cycle leg, the 'moderate to high intensity exercise' equations proposed by Jeukendrup and Wallis[257] were chosen as exercise was undertaken at 70% $\dot{V}O_{2peak}$:

***Equation 3.2* - Substrate oxidation during 'moderate to high intensity exercise'.**

$$CHO = \left(\left[4.210 \times \dot{V}_{CO_2}\right] - \left[2.962 \times \dot{V}_{O_2}\right]\right) \times 60$$

$$Fat = \left(\left[1.695 \times \dot{V}_{O_2}\right] - \left[1.701 \times \dot{V}_{CO_2}\right]\right) \times 60$$

CHO, carbohydrate.

Energy yield of each substrate was taken as 4.07 kcal/g for CHO (assumption of 20% glucose, 80% glycogen contribution) and 9.75 kcal/g for fat[257]. CHO and fat oxidation rates were multiplied by their energy yields and these values added together to calculate EE.

Substrate oxidation calculation under either KME condition was not possible[69] (2.3.6), nor were they possible during the 10 km run as a metabolic/ventilatory steady state could not be assumed[391].

3.2.8 Subjective Measures

Generalised (Borg) and Localised Ratings of Perceived Exertion (RPE), Gastrointestinal (GI) Distress, and Appetite measures (*Figure 3.3)* were assessed as described in General Methods 2.3.4.

3.2.9 Blinding Efficacy

Participants were asked to try and identify each of the study conditions after they had completed all visits to assess drink-composition blinding effectiveness. They were requested to identify each visit based on running performance alone (ranking the visits from perceived 'fastest to slowest runs'), drink taste alone ('from the taste and palatability only'), and overall impression ('from the overall experience, incorporating drink taste, effort and GI perceptions during the cycle and run, overall exercise performance, etc'). They graded each selection with a certainty rating on a linear scale from 0 (completely uncertain) to 10 (completely certain).

3.2.10 Data Analysis

Outcome Measures

The primary outcome for this study was time-to-complete for the 10 km running TT. Secondary outcomes included measures of expired gas composition & ventilation, HR, circulating metabolites, RPE, appetite, and GI distress.

Sample Size Calculation

A sample size calculation was performed in G*Power[380] with 10 km TT time as the primary outcome measure. There is no existing data on the effect of KME supplementation on bike-to-run performance,

therefore a f = 1.130 (Cohen's d[392] = 2.260) was used based on Cox et al.[107]. Alongside this a f = 1.024-1.450 was calculated from the smallest worthwhile difference[393] (SWD) for a preloaded 10 km TT protocol being taken as 2.1%[394], with an expected coefficient of variation (CV) of 1-2%[386]. With a significance level (α) of 0.05 and power (1-β) of 0.8 (3 conditions, 1 measurement per condition) a sample size of 6-9 was required. This study aimed to recruit 14-16 participants to account for uncertainty of observed effects (findings in Cox et al.[107] used fasted participants and were not subsequently replicated[31,33,146,154] so translatability was uncertain) and to allow for attrition.

Dataset Sample Sizes

Plasma biochemistry data is n = 7 due to a cannula failure for one participant. Plasma metabolomics data is n = 10 (with one participant missing the High-KME visit, and one missing the Low-KME visit), and plasma hormone data is n = 8 as High-KME and PLA blood samples were collected for the participant with the cannula failure (which occurred during their Low-KME visit), see *Figure 3.1*. All n values are reported on applicable figures and tables.

Area-Under-The-Curve

Area-under-the-curve (AUC) was calculated for selected metrics from -60 min onwards.

Statistics

Statistics were performed as described in 2.5. 'Condition-level' refers to post-hoc analyses justified by a significant condition effect, thus reflecting comparisons between the averages of each of the three conditions (i.e. time collapsed). Differences between conditions at specific timepoints, justified by a significant interaction effect, are represented graphically, rather than in text, in all instances except for when only a trend for significance was present. Simple linear regressions or Pearson's correlations were performed where appropriate between Δ*metric* values (the difference between KME and PLA values, as KME values *minus* PLA) for two metrics, with High-KME and Low-KME data pooled (i.e. 'High-

KME *minus* PLA' and 'Low-KME minus PLA' values in a single dataset). Regressions/correlations between ΔHigh-KME and ΔLow-KME were also assessed for a selection of single metrics.

Associations between NMR plasma biomarkers and plasma βHB concentration were determined by linear regression modelling, accounting for gender. Post-hoc p-value adjustments were made to correct for multiple comparisons using Benjamini-Hochberg false discovery rate procedure[395]. Data are presented as 1-SD increment in biomarker concentration versus unit βHB concentration at three timepoints: Fasted, -10 min, and +60 min. SD was used in lieu of SEM as it is standard for linear regression analyses of this type[395]. A total of 179 biomarkers were measured, split into lipoprotein and non-lipoprotein biomarker sub-analyses. The non-lipoprotein sub-analysis presented here contained 68 direct measurements & 11 ratios of these measurements.

3.3 Results

3.3.1 Pilot Work Results

A full protocol pilot (n = 2) established that, whilst the prescribed intensity of the cycle leg was suitable and KME/PLA drinks were well tolerated, consuming Drink 1 (*Table 3.5*) 40 min before the start of the cycle leg (-40 min) did not allow sufficient time for plasma [βHB] to plateau (*Figure 3.4*) before exercise commencement. This ran counter to the study aim of establishing consistent between-condition differences in circulating levels of βHB.

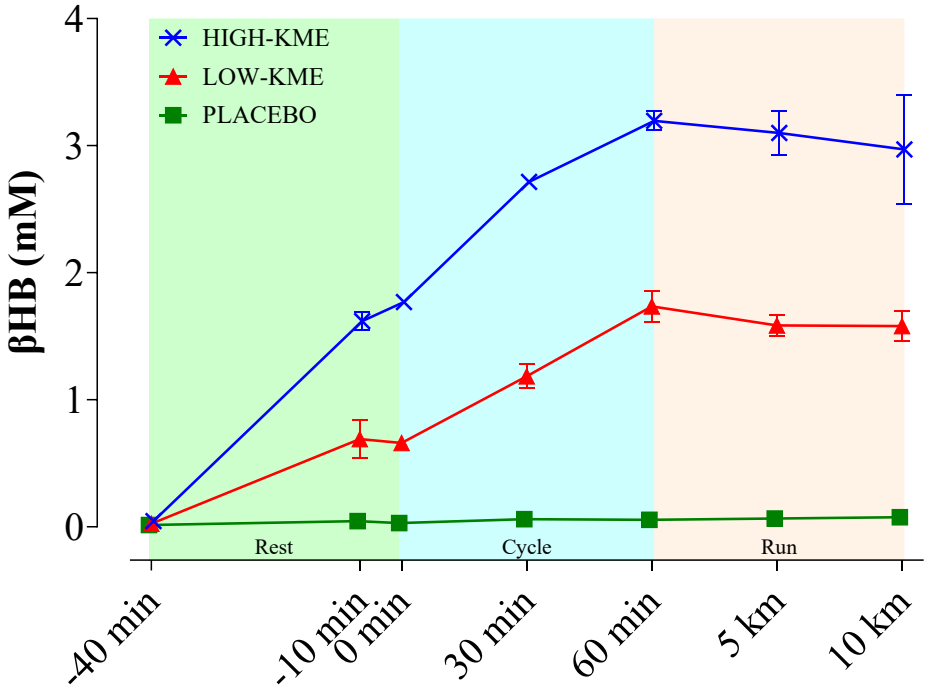


***Figure 3.4* - Pilot study plasma βHB at rest, during 70% $\dot{V}O_{2peak}$ cycling, and during the 10 km running TT in the Placebo, Low-KME, and High-KME conditions.**
Data presented as mean ± SEM in mM. n = 2.

Resting plasma [βHB] kinetics were subsequently explored with a n = 5 sub-pilot. Results confirmed that 40 min was insufficient for plasma [βHB] to plateau in this larger sample size, with substantial inter-individual variation noted, but that 60 min was sufficient (results are found in Appendix E). Therefore, the timing for Drink 1 was adjusted from -40 min to -60 min for the main study (3.2.5), with the breakfast remaining at -120 min.

3.3.2 Plasma Biochemistry

No between-condition differences were found for any plasma metabolites when fasted at the commencement of each visit ($p \geq 0.204$; Appendix F).

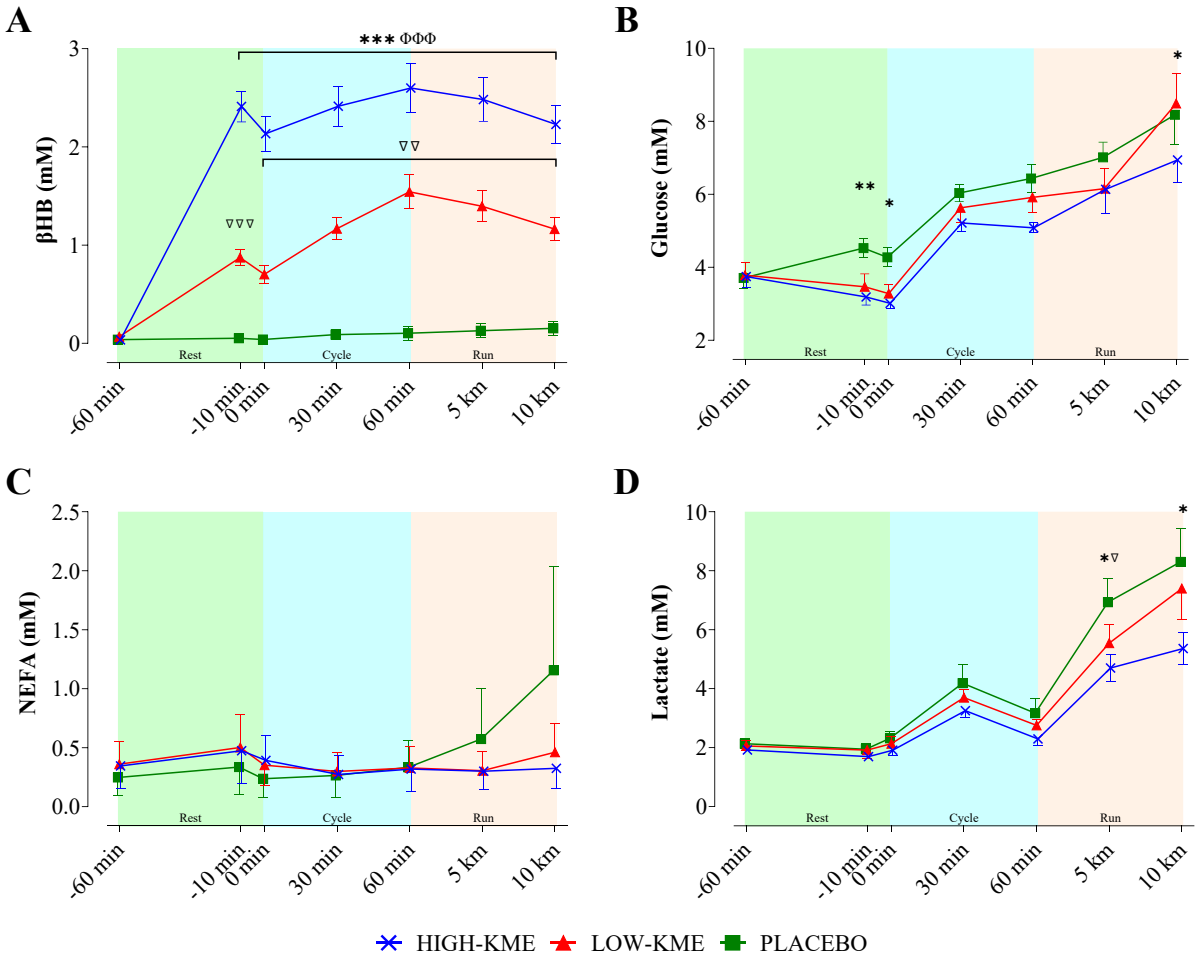


***Figure 3.5* - Plasma metabolites at rest, during 70% $\dot{V}O_{2peak}$ cycling, and during the 10 km running TT in the Placebo, Low-KME, and High-KME conditions.**

*A, βHB (mM); **B**, lactate (mM); **C**, glucose (mM); **D**, NEFA (mM) concentrations. First KME/PLA drink consumed immediately after -60 min timepoint. Data presented as Mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ between High-KME and Placebo conditions. ΦΦΦ $p < 0.001$ between High-KME and Low-KME conditions. ▽ $p < 0.05$, ▽▽ $p < 0.01$, ▽▽▽ $p < 0.001$ between Low-KME and Placebo conditions. n = 7.*

Ingestion of the KME drinks put participants into a state of ketosis in a dose-dependent manner. Effects of time and condition (i.e. dose), as well as a time-x-condition interaction, were found for plasma βHB concentration ($p < 0.001$; *Figure 3.5-A*). From Drink 1 being consumed (following the -60 min sample

being collected) onwards, βHB concentrations were approximately twofold greater in High-KME (2.38 ± 0.20 mM; mean ± SEM) vs Low-KME (1.14 ± 0.16 mM), with levels for PLA not rising above baseline (0.09 ± 0.06 mM). Post-hoc analyses revealed that all between-condition condition-level differences (High-KME vs Low-KME, High-KME vs PLA, Low-KME vs PLA) were significant ($p < 0.001$). There was also an effect of dose on βHB-AUC ($p < 0.001$) with post-hoc analyses showing significant differences between all conditions (High-KME: 6.24 ± 0.51, Low-KME: 3.04 ± 0.27, PLA: 0.25 ± 0.15 mM·hr; $p < 0.001$; *Figure 3.6-A*), indicating distinct condition-dependant βHB exposures. ΔβHB-AUC (Δ represents High-KME-AUC or Low-KME *minus* PLA-AUC values for each participant) was not significantly predicted by participant age, weight, or $\dot{V}O_{2peak}$ ($p \geq 0.246$). ΔβHB-AUC did not predict ΔAUC for any other plasma metabolite or cardiorespiratory measures for 'High-KME *minus* PLA' alone, 'Low-KME *minus* PLA' alone, or combined datasets ($p \geq 0.113$). High-KME and Low-KME ΔβHB-AUCs positively predicted each other's variances ($R^2 = 0.774$; $p = 0.009$).

Effects of time and conditions, alongside an interaction effect, were established for plasma glucose ($p \leq 0.044$; *Figure 3.5-B*), with post-hoc comparisons revealing at a condition-level glucose concentrations were lower under High-KME compared to PLA ($p = 0.018$). Trends, or weak trends, were present for glucose being lower under High-KME than PLA at 60 min ($p = 0.055$) and under Low-KME compared to PLA at -10 min ($p = 0.084$). Similarly, a condition effect was seen for plasma glucose-AUC ($p = 0.012$; *Figure 3.6-B*), with levels reduced under High-KME compared to PLA ($p = 0.017$). Δglucose-AUC positively predicted Δlactate-AUC (*Figure 3.7*) and ΔHR-AUC ($R^2 = 0.353$; $p = 0.025$), whilst negatively predicting ΔNEFA-AUC ($R^2 = 0.332$; $p = 0.025$).

No effects of time or condition, nor an interaction effect, were seen for plasma NEFA ($p \geq 0.159$; *Figure 3.5-C*), with no effect of dose observed for NEFA-AUC ($p = 0.761$; *Figure 3.6-C*).

Plasma lactate exhibited time, condition, and interaction effects ($p \leq 0.003$; *Figure 3.5-D*) with post-hoc tests establishing High-KME to be lower than both Low-KME ($p = 0.047$) and PLA ($p = 0.019$) at a condition-level. Trends were present for lactate being lower under High-KME compared to Low-KME at 0 min, 60 min, and 10 km ($p \leq 0.088$) specifically. An effect of condition was seen for lactate-AUC ($p = 0.005$; *Figure 3.6-D*) with High-KME being lower than Low-KME ($p = 0.049$) or PLA ($p = 0.023$),

indicating that circulating lactate levels were suppressed proportionate to βHB levels. Δlactate-AUC and ΔNEFA-AUC negatively predicted each other's variances ($R^2 = 0.347$; $p = 0.027$).

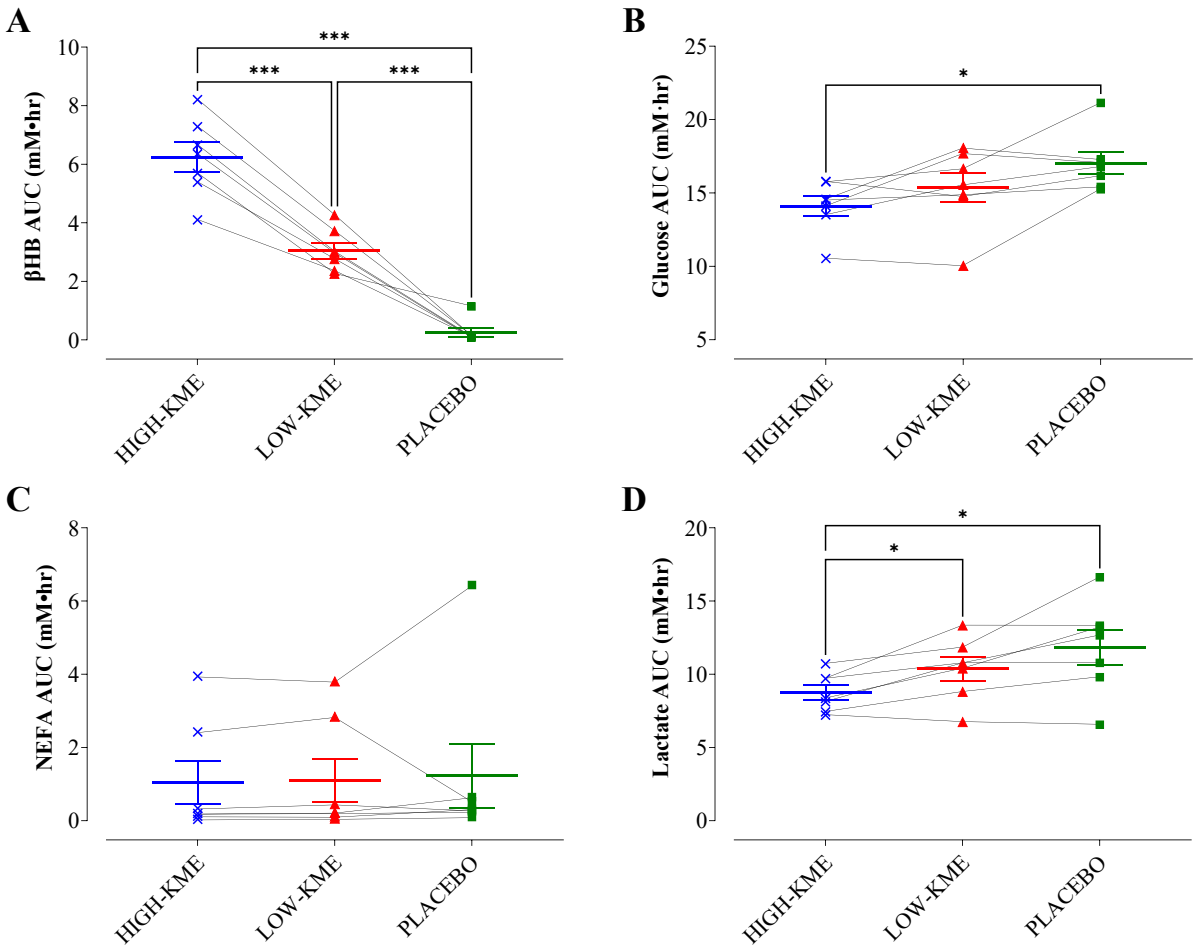


***Figure 3.6* - Plasma metabolite AUCs at rest, during 70% $\dot{V}O_{2peak}$ cycling, and during the 10 km running TT in the Placebo, Low-KME, and High-KME conditions.**

***A**, βHB (mM·hr); **B**, lactate (mM·hr); **C**, glucose (mM·hr); **D**, NEFA (mM·hr). AUC, area-under-the-curve. Data presented as Mean ± SEM with individual values plotted. * $p < 0.05$, *** $p < 0.001$ between conditions. $n = 7$.*

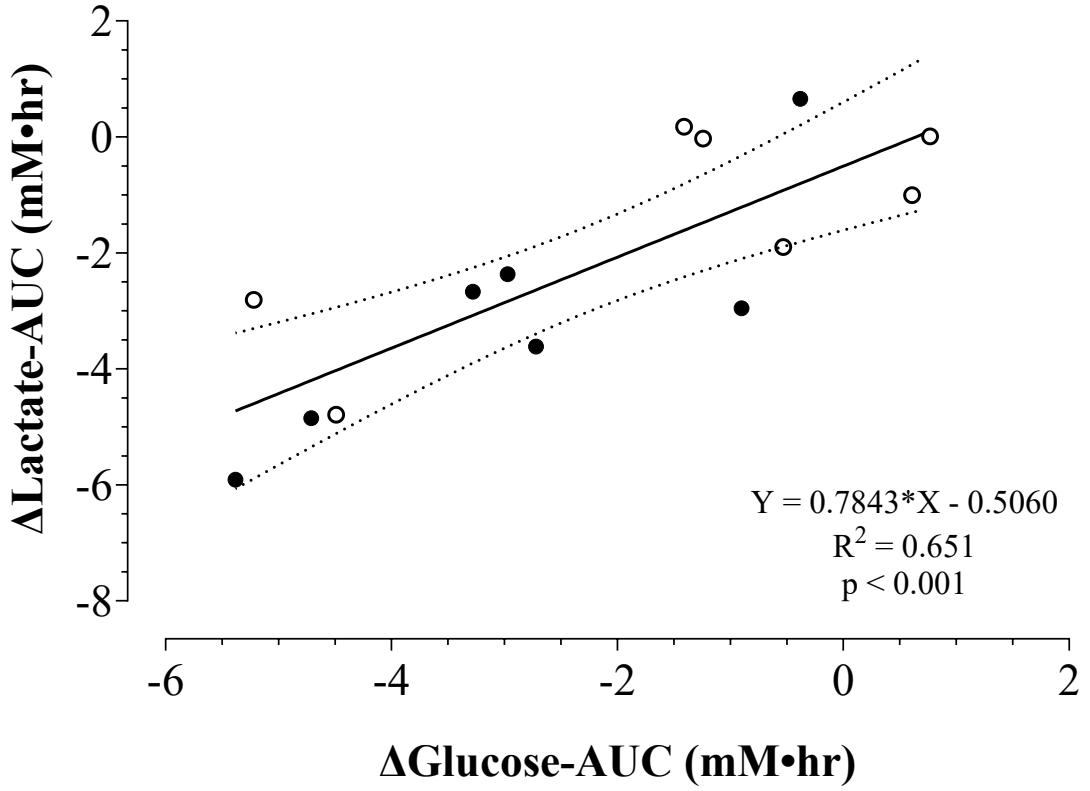
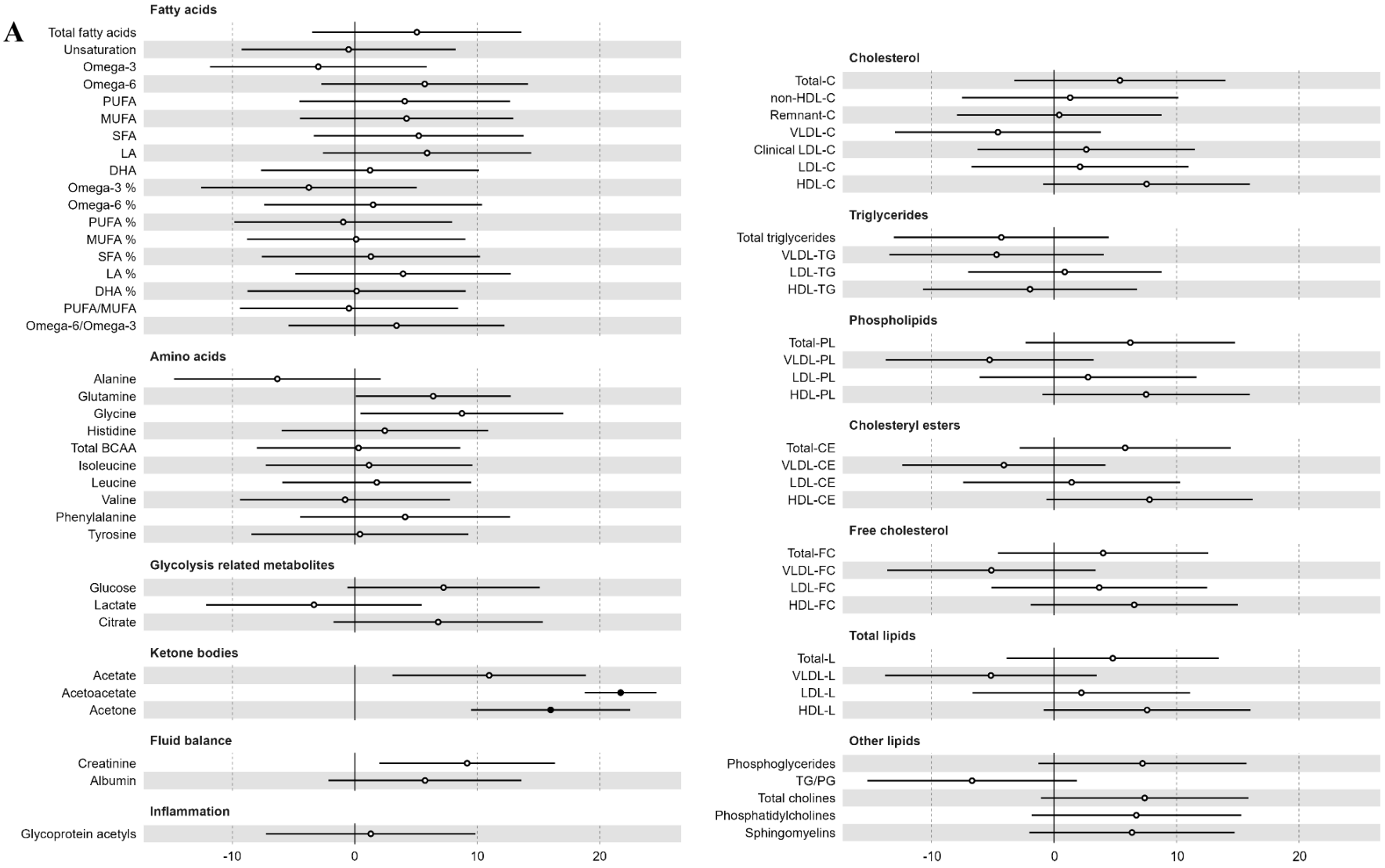


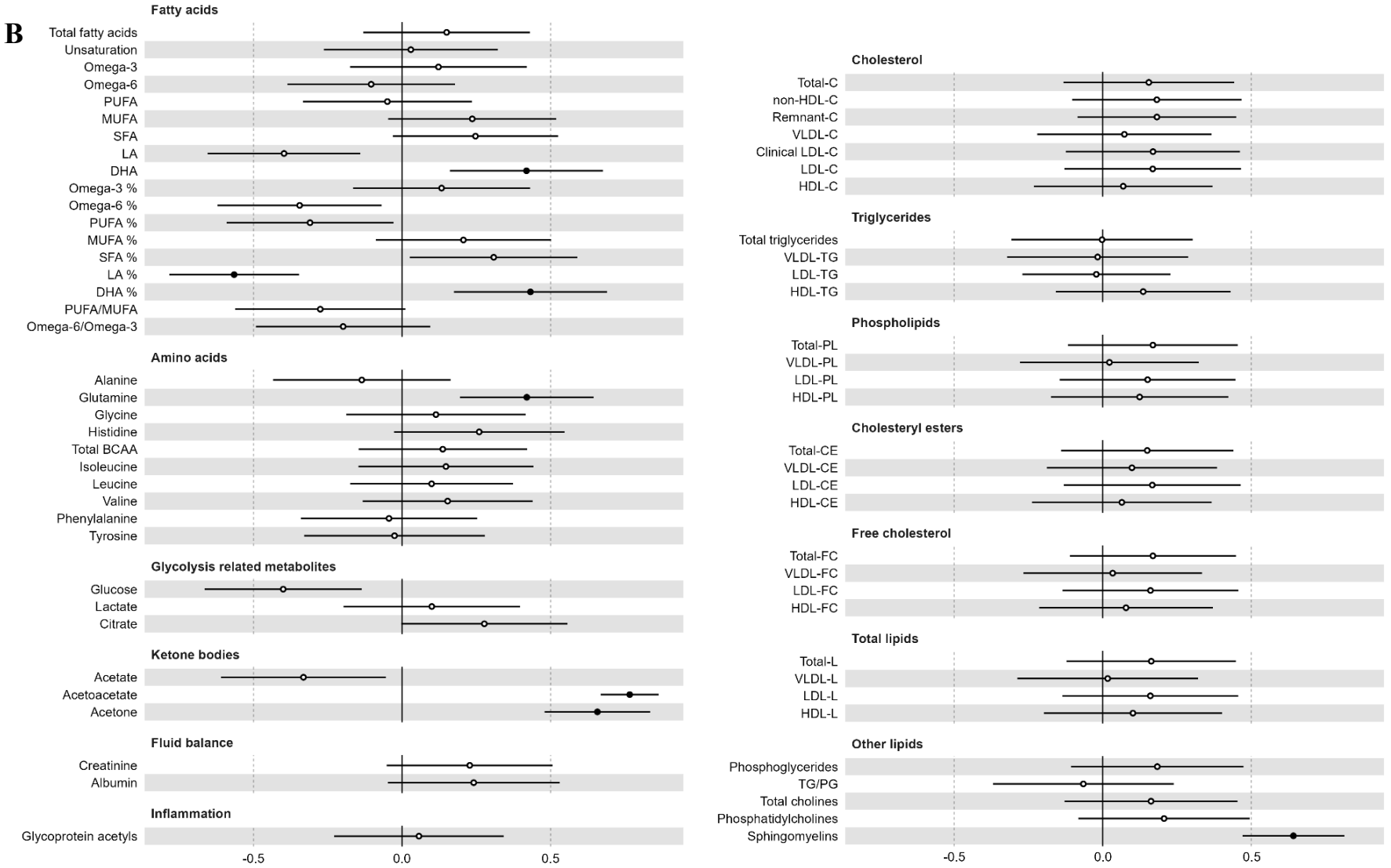
***Figure 3.7* - ΔGlucose-AUC vs ΔLactate-AUC.**

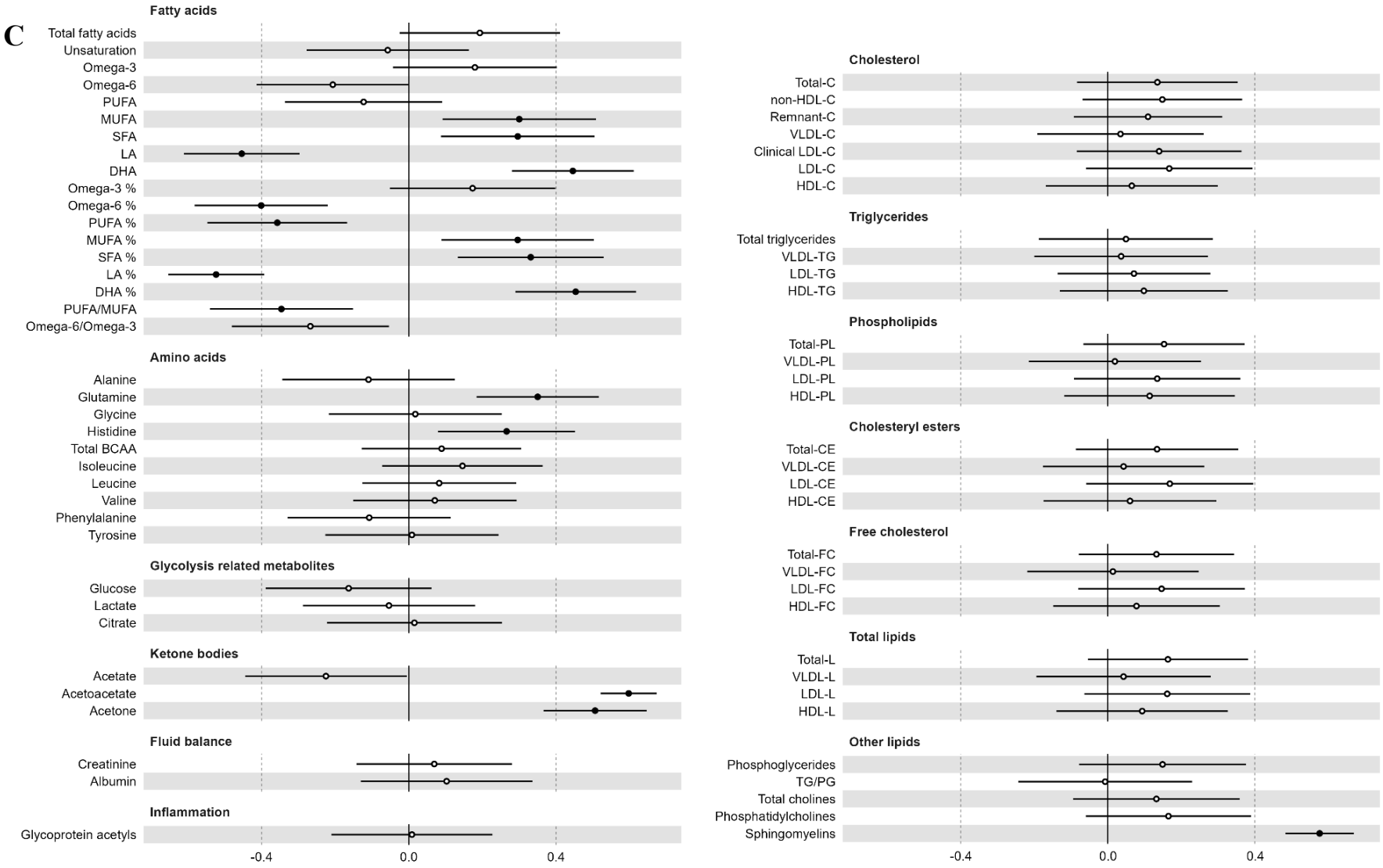
Area-under-the-curve (AUC) calculated from -60 min to completion of the 10 km run leg, in mM·hr. Δ, between condition difference calculated as High-/Low-KME minus PLA. Open circles, Δ of Low-KME minus PLA; closed circles, Δ of High-KME minus PLA. Solid line, linear best fit; dashed lines, 95% confidence intervals. n = 7.

Plasma Metabolomics



1-SD increment in biomarker concentration per unit increment in βHB concentration


B
Fatty acids
Total fatty acids
Unsaturation
Omega-3
Omega-6
PUFA
MUFA
SFA
LA
DHA
Omega-3 %
Omega-6 %
PUFA %
MUFA %
SFA %
LA %
DHA %
PUFA/MUFA
Omega-6/Omega-3
Amino acids
Alanine
Glutamine
Glycine
Histidine
Total BCAA
Isoleucine
Leucine
Valine
Phenylalanine
Tyrosine
Glycolysis related metabolites
Glucose
Lactate
Citrate
Ketone bodies
Acetate
Acetoacetate
Acetone
Fluid balance
Creatinine
Albumin
Inflammation
Glycoprotein acetyls
-0.5
0.0
0.5
Cholesterol
Total-C
non-HDL-C
Remnant-C
VLDL-C
Clinical LDL-C
LDL-C
HDL-C
Triglycerides
Total triglycerides
VLDL-TG
LDL-TG
HDL-TG
Phospholipids
Total-PL
VLDL-PL
LDL-PL
HDL-PL
Cholesteryl esters
Total-CE
VLDL-CE
LDL-CE
HDL-CE
Free cholesterol
Total-FC
VLDL-FC
LDL-FC
HDL-FC
Total lipids
Total-L
VLDL-L
LDL-L
HDL-L
Other lipids
Phosphoglycerides
TG/PG
Total cholines
Phosphatidylcholines
Sphingomyelins
-0.5
0.0
0.5
1-SD increment in biomarker concentration per unit increment in βHB concentration



1-SD increment in biomarker concentration per unit increment in βHB concentration

***Figure 3.8* - Associations between NMR plasma biomarkers and plasma βHB concentrations.**
*Timepoints: **A**, fasted. **B**, -10 min (postprandial, post-first KME/PLA drink, resting). **C**, 60 min (postprandial, post all KME/PLA drinks, end of 1 hr cycle leg). Effect estimate is a 1-SD increase in metabolite concentration for each unit increase in [βHB]. Closed circles, significant associations (false discovery rate-corrected[395]). BCAA, branched-chain amino acids; βHB, beta-hydroxybutyrate; C, cholesterol; CE, cholesteryl esters; DHA, docosahexaenoic acid; FC, free cholesterol; HDL, high-density lipoprotein; LA, linoleic acid; LDL, low-density lipoprotein; Omega 3, omega 3 fatty acids; Omega 6, omega 6 fatty acids; MUFA, monounsaturated fatty acids; PG, phosphoglycerides; PL, phospholipid; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; TG, triglyceride; VLDL, very-low-density lipoprotein; Fatty acid %, ratio of specific fatty acid type to total fatty acids; XXX/YYY, ratio between biomarkers presented. n = 10.*

Metabolomics analyses revealed that both at rest (-10 min) and after 1 hr steady state cycling exercise (+60 min), plasma βHB levels significantly positively correlated with sphingomyelins, docosahexaenoic acid (DHA; as absolute concentration and as a proportion of total free FAs [%FA]), glutamine, AcAc, and acetone, and was negatively associated with linoleic acid (LA) %FA (*Figure 3.8-B/C*). Additionally, during exercise only (+60 min; *Figure 3.8-C*), monounsaturated fatty acids (MUFA; absolute and %FA), saturated fatty acids (SFA; absolute and %FA), and histidine were positively associated with βHB concentration. Absolute levels of LA, Omega-6 %FA, polyunsaturated fatty acids (PUFA) %FA, and the PUFA-to-MUFA ratio were all negatively associated with plasma βHB level at +60 min only. When overnight fasted (baseline state; *Figure 3.8-A*), only AcAc and acetone positively correlated with βHB concentration reflecting baseline ketogenesis and ketolysis.

No correlations were found between plasma βHB level and any lipoprotein biomarkers at any timepoint (Appendix G).

3.3.3 10 km Run Time Trial

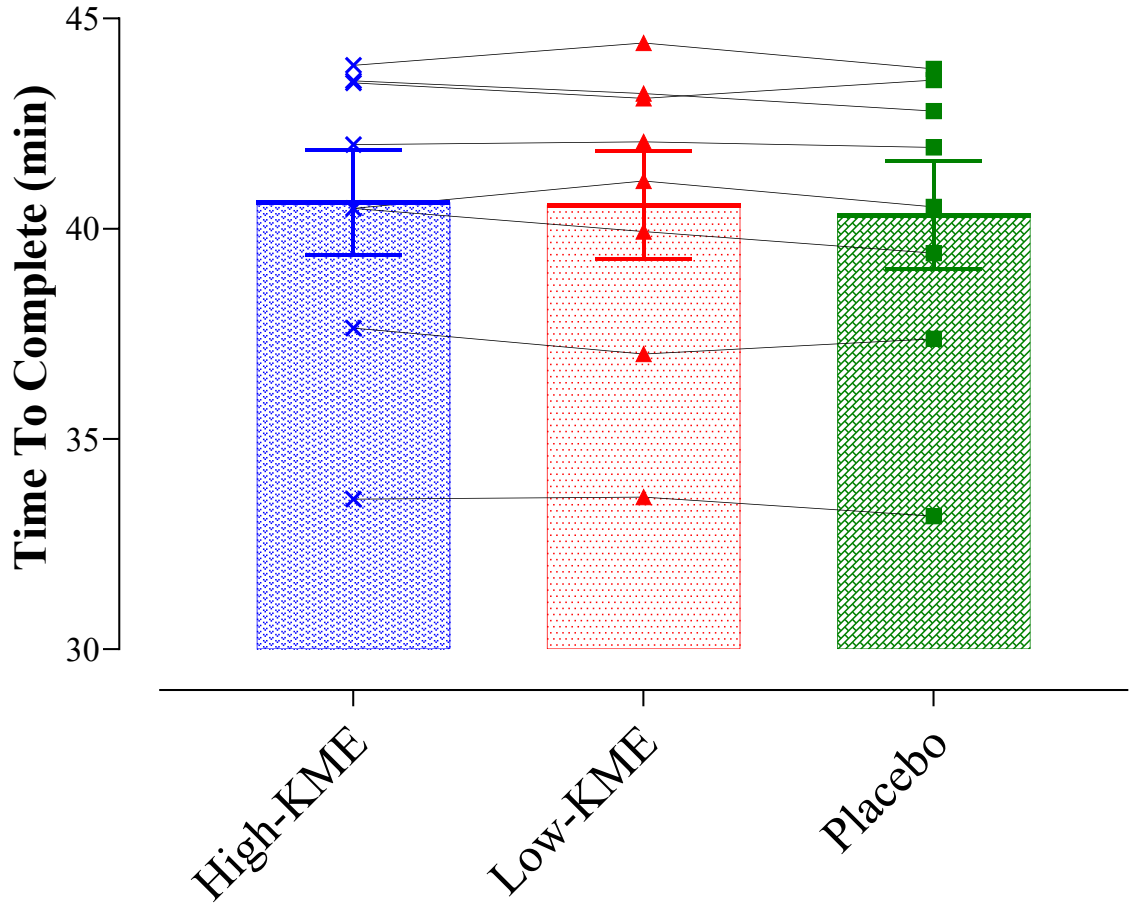


***Figure 3.9* - Effect of KME dose on 10 km running TT performance in the Placebo, Low-KME, and High-KME conditions.**

Data presented as Mean ± SEM with individual values plotted. n = 8.

Time to completion for the 10 km run TT was not different between conditions (High-KME: 40.63 ± 1.25, Low-KME: 40.56 ± 1.28, PLA: 40.32 ± 1.28 min; p = 0.144; *Figure 3.9*). Only one participant exhibited a between-dose time difference exceeding the pre-established SWD threshold of 2.1%, with their PLA time being 2.6% faster than High-KME. ΔTT time was not predicted by ΔβHB-AUC or ΔNEFA-AUC nor any cardiorespiratory measure ($p \geq 0.113$). It was however negatively predicted by Δglucose-AUC ($R^2 = 0.011$; $p = 0.043$) and Δlactate-AUC ($R^2 = 0.299$; $p = 0.043$) though in both instances this only explained a small proportion of the observed variance.

No order effect was observed for 10 km run performance (first visit: 40.44 ± 1.27, second: 40.51 ± 1.24, final: 40.56 ± 1.31 min; p = 0.759), indicating that there were no substantial learning effects or fitness changes at a group level present across the study period.

3.3.4 Cardiorespiratory Measures

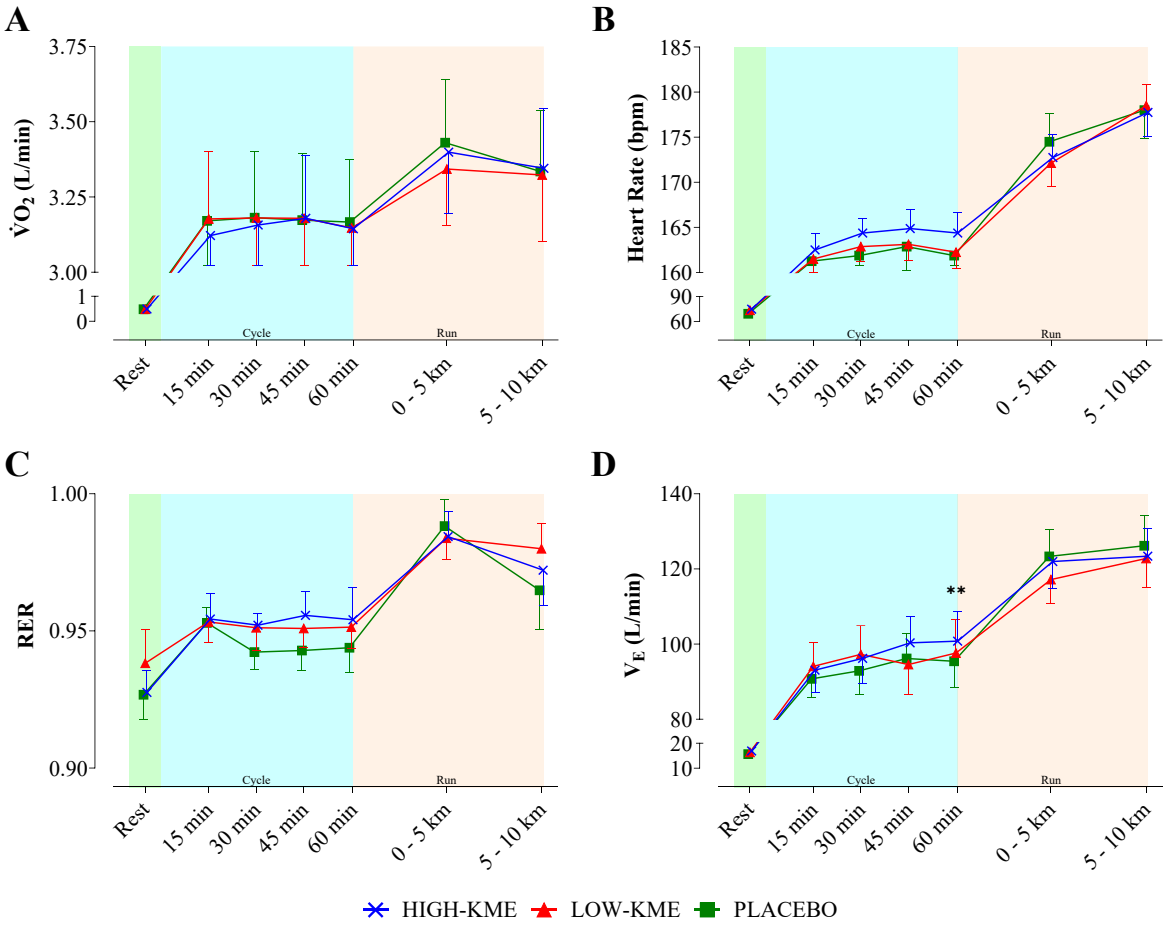


***Figure 3.10* - Cardiorespiratory measures at rest, during 70% $\dot{V}O_{2peak}$ cycling, and during the 10 km running TT in the Placebo, Low-KME, and High-KME conditions.**

***A,** oxygen utilisation ($\dot{V}O_2$) in litres per minute (L/min); **B,** heart rate in beats per minute (bpm); **C,** respiratory exchange ratio (RER; $\dot{V}CO_2/\dot{V}O_2$); **D,** minute ventilation (V_E) in litres per minute (L/min). Collected at rest, during the cycle leg (11-15/26-30/41-45/56-60 min), and throughout the run leg TT (0-5/5-10 km). ** p < 0.01 between High-KME and PLA conditions. Data presented as Mean ± SEM. n = 8.*

Effects of time were established for $\dot{V}O_2$, HR, RER, V_E, $\dot{V}CO_2$, V_T, and BF ($p \leq 0.002$; *Figure 3.10* and *Table 3.6*). No measures exhibited condition effects ($p \geq 0.207$), whilst $\dot{V}O_2$, $\dot{V}CO_2$, RER, and V_T did not exhibit time-x-condition interaction effects ($p \geq 0.380$). Significant interaction effects were present,

however, for HR, BF, and V_E ($p \leq 0.043$). Post-hoc analyses did not reveal any between-condition differences at a condition-level ($p \geq 0.104$). Weak trends were found for BF being greater under High-KME compared to PLA at Rest ($p = 0.089$), and for V_E under both High-KME and PLA being greater than during Low-KME across 0-5 km ($p \leq 0.86$).

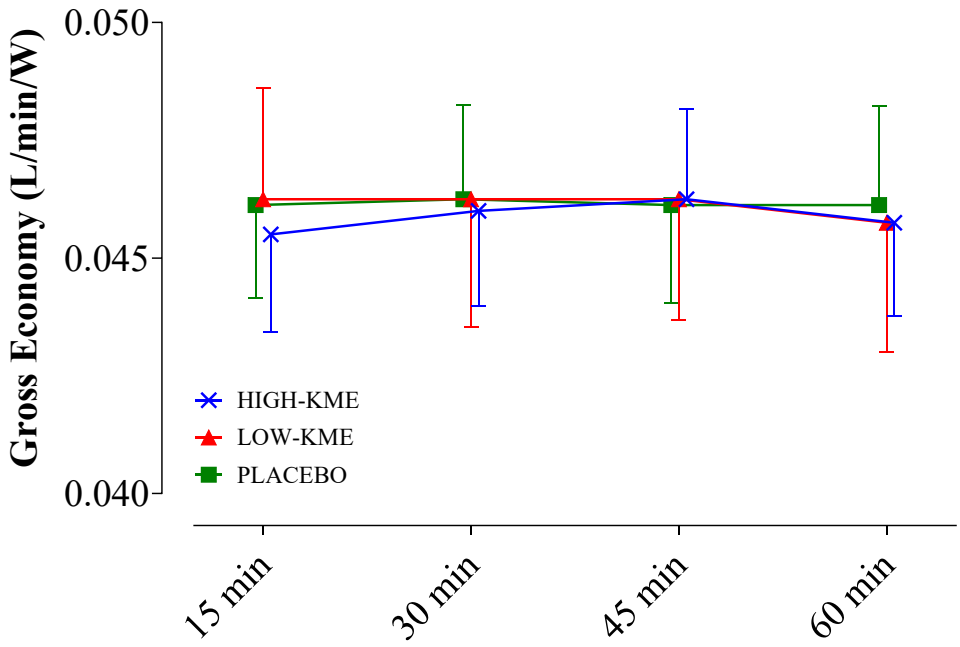


***Figure 3.11* - 70% $\dot{V}O_{2peak}$ cycling gross economy in the Placebo, Low-KME, and High-KME conditions.**

As litres of oxygen utilised per minute per watt (L/min/W). Collected across 4-minute sampling periods during the cycle leg (11-15 min/26-30 min/41-45 min/56-60 min). Data presented as Mean ± SEM. n = 8.

Gross economy, across the cycle leg where a metabolic steady state could be assumed[396], did not exhibit time or condition effects ($p \geq 0.650$; *Figure 3.11*). Though a trend for an interaction effect was present ($p = 0.063$), exploratory post-hoc tests did not reveal condition effects at any timepoint ($p \geq 0.569$).

Statistical outcomes did not differ when $\dot{V}O_2$ and $\dot{V}CO_2$ were scaled by bodyweight.

***Table 3.6* - Cardiorespiratory measures at rest, during 70% $\dot{V}O_{2peak}$ cycling, and during the 10 km running TT in the Placebo, Low-KME, and High-KME conditions.**

	Dose	Rest	11-15 min	26-30 min	41-45 min	56-60 min	0-5 km	5-10 km	p Time	p Dose	p Interaction
$\dot{V}CO_2$ (L/min)	High-KME	0.46 ± 0.04	2.97 ± 0.18	3.00 ± 0.19	3.03 ± 0.19	3.00 ± 0.21	3.34 ± 0.19	3.24 ± 0.17			
	Low-KME	0.45 ± 0.04	3.03 ± 0.21	3.03 ± 0.23	3.03 ± 0.23	3.00 ± 0.25	3.29 ± 0.19	3.26 ± 0.22	<0.001	0.974	0.639
	Placebo	0.45 ± 0.05	3.02 ± 0.19	3.00 ± 0.20	2.99 ± 0.20	2.99 ± 0.20	3.39 ± 0.21	3.22 ± 0.20			
V_T (L)	High-KME	0.84 ± 0.04	2.45 ± 0.19	2.29 ± 0.18	2.31 ± 0.17	2.23 ± 0.17	2.14 ± 0.15	2.12 ± 0.15			
	Low-KME	0.88 ± 0.11	2.47 ± 0.22	2.37 ± 0.21	2.31 ± 0.21	2.23 ± 0.20	2.19 ± 0.16	2.14 ± 0.16	<0.001	0.648	0.666
	Placebo	0.85 ± 0.08	2.46 ± 0.19	2.35 ± 0.20	2.31 ± 0.19	2.21 ± 0.17	2.17 ± 0.15	2.03 ± 0.14			
BF (/min)	High-KME	19.9 ± 1.0	38.5 ± 1.5	42.6 ± 2.4	43.9 ± 2.4	45.5 ± 2.4	57.6 ± 2.6	59.3 ± 3.7			
	Low-KME	19.3 ± 1.2	38.8 ± 1.6	41.7 ± 2.2	41.6 ± 2.5	44.2 ± 2.9	54.7 ± 3.1	58.7 ± 3.8	<0.001	0.207	0.043
	Placebo	18.5 ± 1.1	37.7 ± 1.9	40.3 ± 2.3	42.4 ± 2.5	43.8 ± 2.7	57.5 ± 2.5	62.7 ± 3.1			

$\dot{V}CO_2$, carbon dioxide production in litres per minute (L/min); V_T, tidal volume in litres (L); BF, breathing frequency in breathes per minute (/min). Collected at rest, during the cycle leg, and throughout each 5 km of the 10 km run leg TT. p, p-value for given main/interaction effect. Data presented as Mean ± SEM. n = 8.

Substrate Oxidation

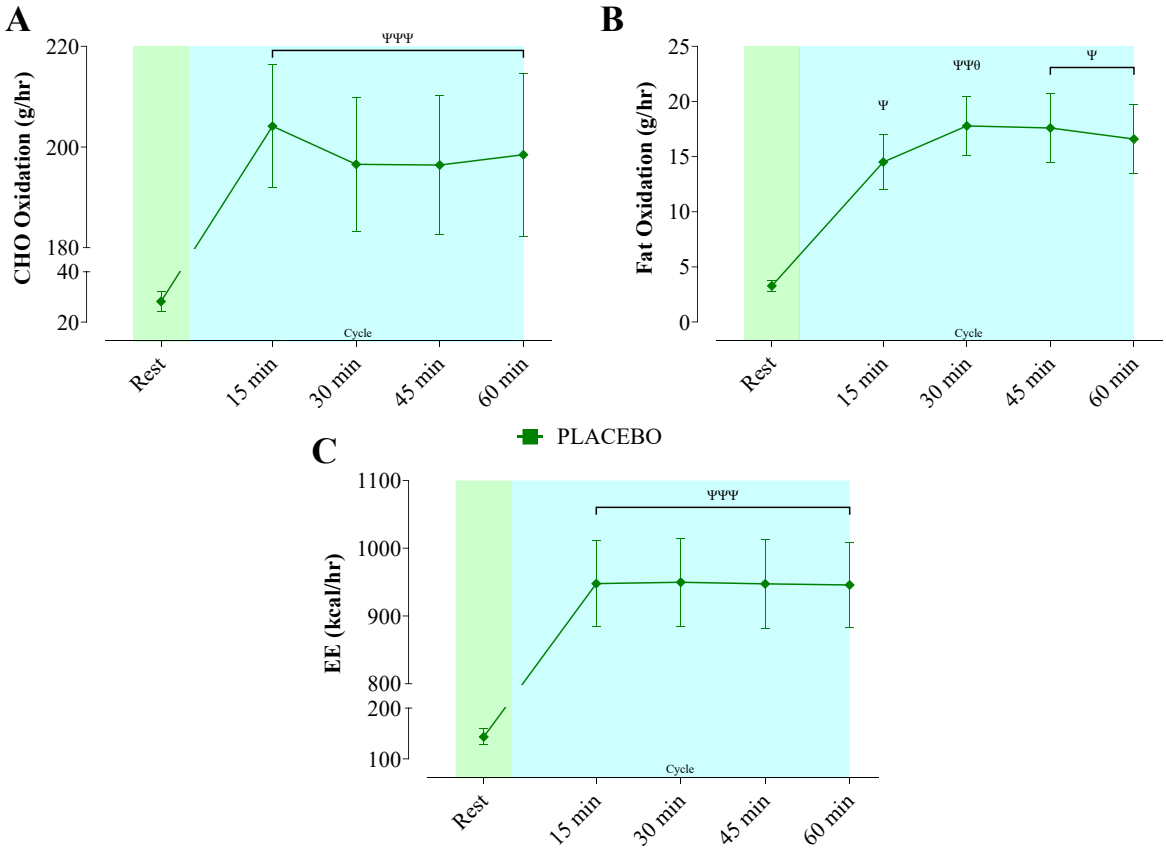


***Figure 3.12* - Substrate oxidation and energy expenditure at rest and during 70% $\dot{V}O_{2peak}$ cycling in PLA only.**

*A, Carbohydrate (CHO) Oxidation Rate (g/hr); **B**, Fat Oxidation Rate (g/hr); **C**, Total Energy Expenditure (EE; kcal/hr). Collected across 4-minute sampling periods at rest and during the cycle leg (11-15 min/26-30 min/41-45 min/56-60 min). Data presented as Mean ± SEM. $^{\Psi\Psi\Psi}$ $p < 0.001$ between timepoint and Rest; $^{\theta}$ $p < 0.05$ between 15 min and 30 min. $n = 8$.*

Taking PLA data in isolation at rest and across the cycle leg, effects of time were found for carbohydrate and fat oxidation, as well as EE ($p < 0.001$; *Figure 3.12*).

3.3.5 Gastrointestinal Distress & Perceived Exertion

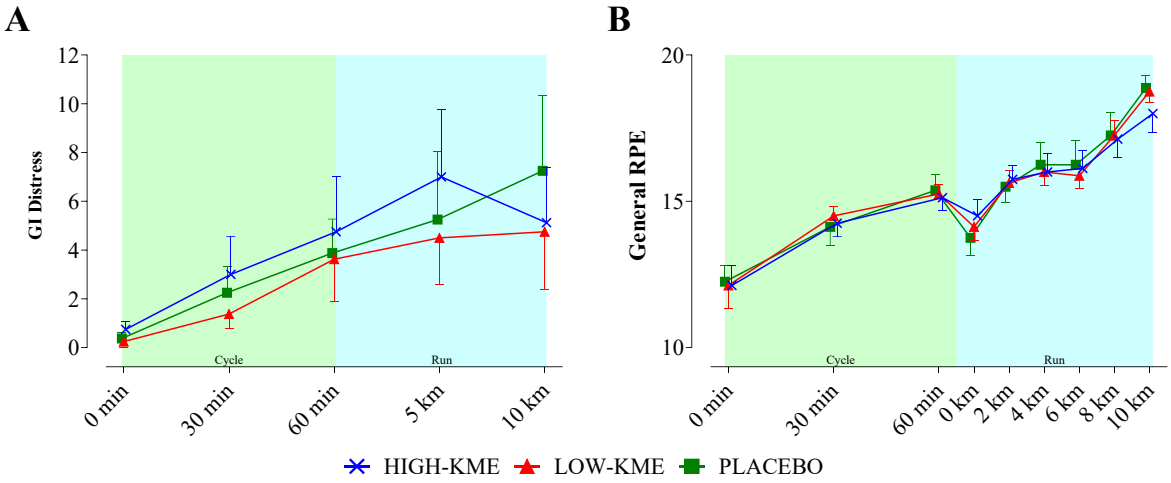


***Figure 3.13* - GI Distress and General RPE during 70% $\dot{V}O_{2peak}$ cycling and the 10 km running TT in the Placebo, Low-KME, and High-KME conditions.**

GI, gastrointestinal; RPE, rating of perceived exertion (6-20 Borg scale). GI Distress calculated as the sum of symptom scores for each of the 12 specific measures at a given timepoint. Data presented as Mean ± SEM. n = 8

Gastrointestinal distress exhibited an effect of time ($p = 0.001$), though no condition or interaction effects were seen ($p \geq 0.119$; *Figure 3.13-A*).

An effect of time was seen for all RPE measures (General: $p < 0.001$, *Figure 3.13-B*; Localised: *Table 3.7*). No condition or interaction effects were seen for any metrics (General: $p \geq 0.810$), however, with the exception of *Anxiety of Breathing* where a weak trend for a condition effect was present and exploratory condition-level post-hocs revealed values to be greater for High-KME than PLA ($p = 0.028$).

***Table 3.7* - Localised RPE metrics during 70% $\dot{V}O_{2peak}$ cycling and during the 10 km running TT in the Placebo, Low-KME, and High-KME conditions.**

Measure	Dose	0 min	30 min	60 min	2 km	10 km	p Time	p Dose	p Interaction
Breathlessness	High-KME	3.1 ± 0.4	4.3 ± 0.8	4.8 ± 0.8	5.8 ± 0.8	8.1 ± 0.5	<0.001	0.964	0.889
	Low-KME	3.3 ± 0.3	3.9 ± 0.4	4.6 ± 0.6	6.0 ± 0.4	8.4 ± 0.7			
	Placebo	3.4 ± 0.4	3.9 ± 0.6	4.8 ± 0.5	6.0 ± 0.6	8.4 ± 0.5			
Anxiety of Breathing	High-KME	1.6 ± 0.6	2.4 ± 0.8	3.3 ± 1.0	4.4 ± 1.2	5.4 ± 1.4	0.004	0.081	0.617
	Low-KME	1.6 ± 0.6	2.1 ± 0.6	3.1 ± 1.0	3.9 ± 1.0	5.1 ± 1.2			
	Placebo	1.6 ± 0.7	2.1 ± 0.7	2.9 ± 1.0	3.0 ± 1.1	5.4 ± 1.3			
Leg Discomfort	High-KME	2.9 ± 0.6	4.1 ± 0.7	4.5 ± 0.8	4.8 ± 0.9	6.8 ± 1.1	<0.001	0.550	0.982
	Low-KME	3.0 ± 0.5	4.4 ± 0.7	5.1 ± 0.8	4.6 ± 0.8	6.9 ± 1.1			
	Placebo	2.6 ± 0.5	4.0 ± 0.7	4.8 ± 0.7	4.3 ± 0.9	6.6 ± 1.1			
Anxiety of Leg Discomfort	High-KME	2.3 ± 0.7	3.1 ± 0.9	3.8 ± 0.9	3.9 ± 0.9	4.6 ± 1.4	0.024	0.326	0.966
	Low-KME	2.4 ± 0.6	3.8 ± 0.8	4.1 ± 0.9	4.3 ± 1.0	5.0 ± 1.2			
	Placebo	1.9 ± 0.6	3.1 ± 0.9	3.8 ± 1.1	3.3 ± 1.0	5.1 ± 1.2			

RPE, rating of perceived exertion. p, p-value for given main/interaction effect. Data presented as Mean ± SEM; 0-10 scale. n = 8.

3.3.6 Appetite

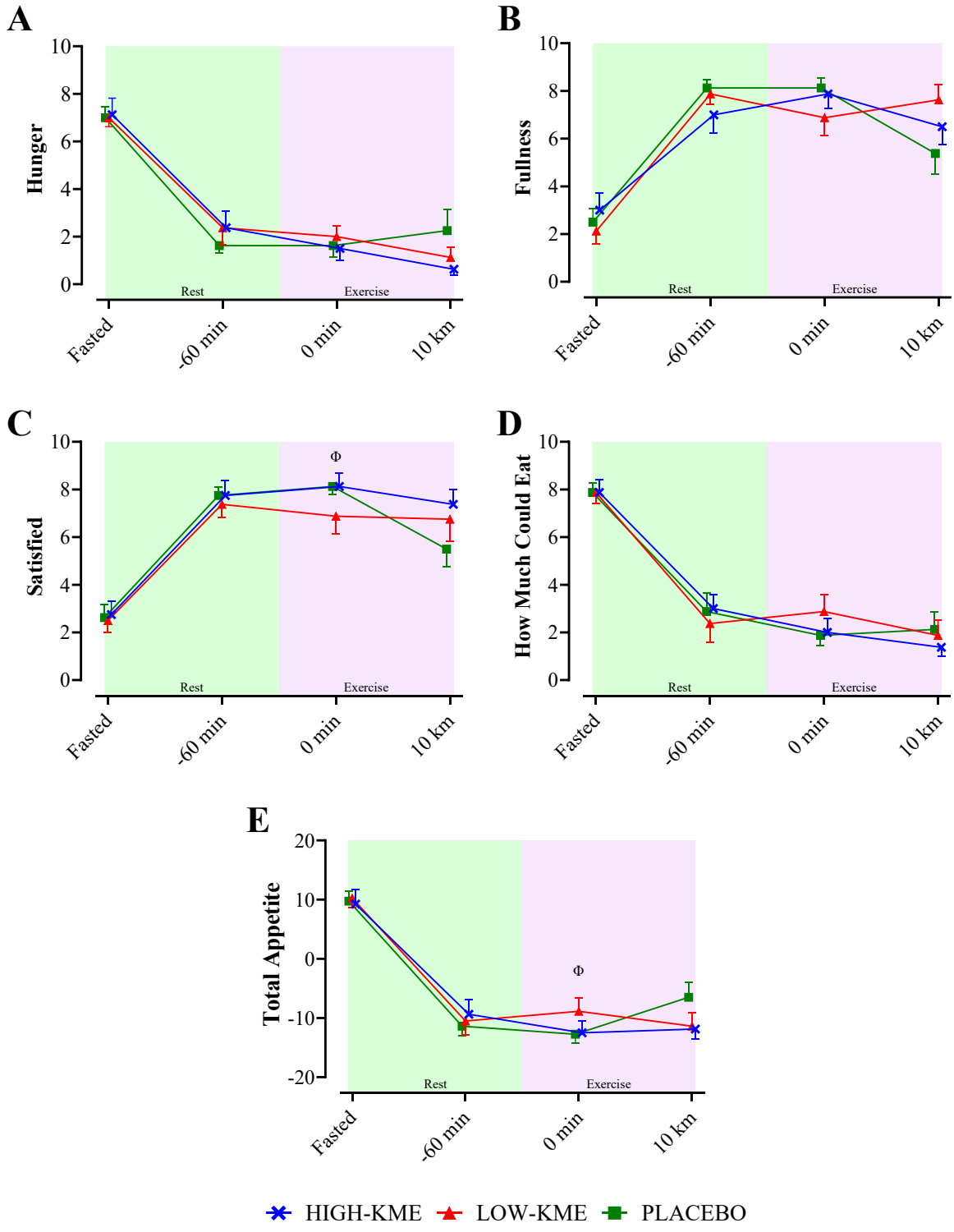


***Figure 3.14* - Subjective appetite measures at rest and during exercise in the Placebo, Low-KME, and High-KME conditions.**

A, *Hunger;* ***B,*** *Fullness;* ***C,*** *Satisfied;* ***D,*** *How Much Could Eat;* ***E,*** *Total Appetite (composite score). Data reported as Mean ± SEM; 0-10 scale (A-D).* $^{\Phi}$ *$p < 0.05$ between High-KME and Low-KME conditions. $n = 8$.*

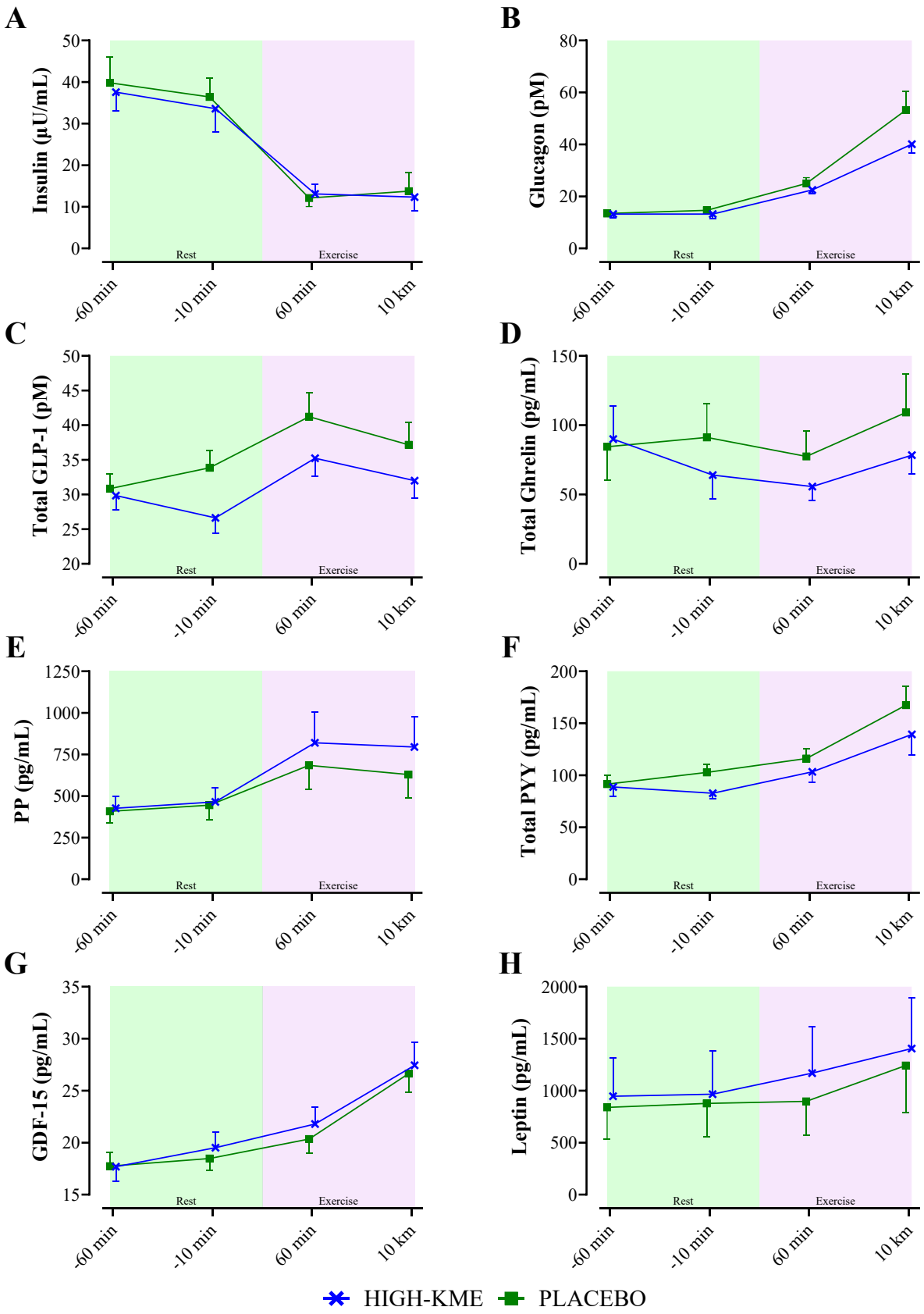


Figure 3.15* - Plasma hormones at rest and during exercise in the Placebo and High-KME conditions.** *A, insulin (µU/mL);* ***B*, glucagon (pM);* ***C****, total glucagon-like peptide-1 (GLP-1; pM);* ***D****, total ghrelin (pg/mL);* ***E,*** *pancreatic polypeptide (PP; pg/mL);* ***F,*** *total peptide YY (PYY; pg/mL);* ***G,*** *growth differentiation factor-15 (GDF-15; pg/mL);* ***H,*** *leptin (pg/mL) concentrations. First KME/PLA drink consumed immediately after -60 min timepoint. Data presented as Mean ± SEM. n = 8.* ***Figure produced, with permission, from raw data provided by Alexander Wythe.***

Subjective Measures

All appetite measures exhibited effects of time ($p < 0.001$; *Figure 3.14*). There was no effect of condition ($p \geq 0.308$) for any metric, but all presented interaction effects ($p \leq 0.037$) with the exception of *How Much Could Eat* ($p = 0.226$).

Plasma Hormones

Analyses were undertaken, with permission, using raw data provided by Alexander Wythe, generated as part of his DPhil research.

Significant effects of time were observed for all appetite/satiety-related plasma hormones ($p \leq 0.031$; *Figure 3.15*), with the exception of total PYY ($p = 0.100$), whilst none exhibited interaction effects ($p \geq 0.134$). Effects of condition were present for total GLP-1 and total PYY ($p \leq 0.035$), alongside weak trends for an effect of condition being seen for glucagon and total ghrelin ($p \leq 0.098$), with levels in each lower under High-KME. Condition effects were not present for any other measures ($p \geq 0.168$).

3.3.7 Blinding Efficacy

Participants correctly identified the visit condition from their perception of the drink taste alone 25% of the time, with an average certainty of 3.2 (0, completely uncertain; 10, completely certain). They identified the dose correctly based on their overall perceptions of the visit 18% of the time, with a 2.0 average certainty. No participants correctly ranked their fastest-to-slowest run times, with an average certainty of 3.8. It can therefore confidently be concluded that blinding for dose-condition was efficacious.

3.4 Discussion

This study sought to investigate whether exogenous ketosis might enhance postprandial multimodal exercise performance in a KME dose-dependent manner, assessed using a 1 hr cycling preload at 70% $\dot{V}O_{2peak}$ immediately followed by a 10 km running TT. Despite plasma βHB being elevated corresponding to KME dose, running performance did not differ between High-KME, Low-KME, or PLA. Circulating βHB concentrations were, however, positively associated with the degree of plasma free FA saturation, and negatively associated with plasma glucose, lactate, total GLP-1, and total PYY.

3.4.1 Circulating βHB Dose-Response

Consumption of the KME elicited a state of exogenous ketosis with dose-dependent elevations to plasma βHB. High-KME saw a rise to 2.14-2.60 mM (average from -10 min onwards: 2.38 mM), whilst Low-KME delivered 0.70-1.54 mM (average: 1.14 mM), with levels in PLA remaining minimal at 0.04-0.15 mM. Though greater variance in concentration was seen across the testing period under Low-KME than in High-KME, the difference between these conditions was >1 mM at all timepoints, with Low-KME always >0.7 mM higher than PLA, maintaining clear between-condition delineations. Therefore, the dosing strategies used permitted interrogation of both the upper and lower thresholds of the 1-3 mM ergogenic range proposed by Evans et al.[24].

The βHB responses observed align with those seen previously for postprandial exercise protocols. Reflecting High-KME, 750 mg/kg KME elevated βHB to 1.5-2.6 mM during intermittent running[151], whilst 918 ± 102 mg/kg[146] and 922 ± 85 mg/kg[157] (both mean ± SD) achieved ~2-3 mM in a pair of studies with the same intermittent cycling protocol. Furthermore, provisioning at 573 mg/kg dose, as used in Low-KME, led to 0.99-1.33 mM during endurance running[154] and ~1-2 mM for treadmill exercise at 54% $\dot{V}O_{2peak}$[150].

The 573 mg/kg provided by Cox et al.[107] to their fasted participants elevated βHB to ~1.75-3 mM, with levels rising to ~2 mM at 20 min after an initial dose of 286.5 mg/kg (573 mg/kg was split 2:1:1 into three drinks, mirroring Low-KME). In juxtaposition, 0.87 ± 0.08 mM was achieved 50 min after the same initial dose in Low-KME, highlighting the influence that the nutritional state of an athlete can have

on βHB kinetics. This adds to limited existing evidence that attainable βHB levels appear to be lower, and potentially slower to peak, when the KME is ingested postprandially compared to fasted. If athletes were to target specific 'optimal'[24] plasma βHB levels when consuming the KME pre-exercise and after a high-carbohydrate meal[5], the interaction between feeding state and βHB profile warrants characterisation to better inform KME dosing and timing. This was therefore interrogated directly in *Chapter 5*.

3.4.2 10 km Running Time Trial Performance

The primary outcome of the work presented here was time to completion for the 10 km treadmill TT. In a postprandial state with optimal in-exercise carbohydrate provision, no performance differences were seen regardless of KME dose. This adds to a body of evidence where no study has seen an enhancement to endurance performance from exogenous ketosis, when assessed as a direct performance outcome (i.e. TT or time-to-exhaustion [TTE], rather than a performance determinant), in a postprandial state[31–33], outside of where the outcome included cognitive elements[231] or when sodium bicarbonate was co-ingested with the KME[157].

In line with my observations, Evans et al.[154] found no effect, in trained runners, on treadmill running 10 km TT time after 1 hr at 65% $\dot{V}O_{2max}$ from 573 mg/kg KME, closely reflecting Low-KME here. This work and mine deviate from Cox et al.'s[107] 2% performance enhancement outcome for the KME despite similarities in physiological demands, with the primary differences being feeding state, as previously discussed, alongside exercise modality. Cox et al.[107] proposed that their ergogenic findings were consequent of a KB-driven shift in oxidative substrate hierarchy, with greater reliance on FAs over glucose resultant of bottlenecked glycolysis via PDH and/or PFK inhibition[65,107,108], evidenced by SM metabolomics data and reduced glycogen depletion. It remains debatable, though, if this mechanism can validly explain high intensity performance enhancement[134], given ketosis-driven 'sparing' of glycogen would reciprocally entail inhibited capacity to utilise said glycogen, spared or otherwise, until KB levels fall. This would, in fact, more likely be ergolytic than ergogenic, with glycogenolysis potentially a limiting factor to exercise capacity in certain settings[5,145,397,398].

Whilst its role is equivocal, it is also unclear if this proposed glycolytic suppression was present in my work. Although plasma lactate levels were lower under High-KME compared to Low-KME and PLA, potentially indicating bottlenecked glycolytic flux[31,65], RER was not reduced indicating that a shift towards FA oxidative dominance did not occur[69]. This may be due to low rates of ketolysis, as KBs are potentially outcompeted as an oxidative substrate when glucose availability is high postprandially, as here, but not when fasted and carbohydrate availability is reduced[137,223,232], as in Cox et al.[107]. Alternatively, in my work KBs themselves could have been oxidised in place of carbohydrate whilst still leaving RER broadly unchanged (RQ, glucose: 1.00, AcAc: 1.00, βHB: 0.89[69]), allowing for glycogen sparing by direct substitution as an oxidative substrate rather than via the Randle cycle[132]. However, if ketolysis were to directly supplant carbohydrate oxidation during the cycle leg at 5.88 g/hr, a rate quantified at a comparable intensity using stable isotope tracers[71], only 6.84 g (3.45%) of the 198.40 g of carbohydrate being utilised on average across this hour (in PLA) would have been spared. Thus, the scope for an ergogenic impact of any substrate-substitution on 10 km performance would likely be minimal[399]. As blood gas measures were not assessed in this chapter, it cannot, however, be discounted that RER in the KME conditions may have been elevated by mild ketoacidosis increasing non-oxidative $\dot{V}CO_2$, which could act to mask a shift towards fatty acid oxidative dominance.

Previous work has also suggested there might exist 'responders' and 'non-responders'[400] to exogenous ketosis[31,154], potentially influenced by SM type I fibre %[71] and sex[31,233,401]. There was no justification for classification in this manner here, with TT times varying by less than the 2.1% SWD[394] for all but one participant, though this work was not powered to explore possible between-sex differences.

3.4.3 Plasma Metabolites

Whilst there was no influence of KME consumption on running performance with either high or low provision, a dose-dependent effect on circulating metabolites was evident.

Glucose & Lactate Metabolism

Attenuation of plasma lactate by up to 50% under exogenous ketosis during exercise has been observed when fasted[71,107,153] and in a postprandial state[151,152,233]. It is not universally observed[125,146,150,154,157,229] however, and there is no clear relationship between the presence/absence of lowered circulating lactate and exercise intensity nor modality. It does appear though that, when present, higher circulating βHB concentrations drive a greater degree of lactate reduction[71,232,233]. Work presented here is the first to directly characterise this dynamic of plasma lactate being lowered proportionate to βHB, with clear suppression seen under High-KME compared to Low-KME and to PLA, but not between Low-KME and PLA. Alongside this, dose-dependent lowering was present during the 10 km run specifically, likely due to lactate levels being markedly elevated compared to the cycle leg, engendering greater scope for ketolytic suppression. As lactate during the run leg was >4 mM, it is unlikely that this lowering was consequent of MCT saturation by KBs slowing efflux from SM[155,156]. It cannot be ruled out, though, that the effect may be in part due to differences in muscle-group recruitment and ventilatory patterns between running and cycling[402], as modality specific responses to the KME are unexplored and circulating lactate kinetics are known to differ between cycling and running[403–405]. Additionally, during exercise a significant proportion of ingested fructose is metabolised into lactate[406], elevating circulating lactate levels compared to glucose alone[407]. Though the effects of exogenous ketones on fructolysis are poorly described, it is possible that lowered plasma lactate under High-KME may have been consequent of ketosis bottlenecking flux through this pathway. However, if this were to be the case, the mechanisms at play are unclear as fructose is primarily metabolised in the liver[408], where ketolytic rates are low[24]. The lack of overall condition effect between Low-KME and PLA, but lowering at 5 km in isolation, suggests that βHB at ~1-1.5 mM is insufficient to robustly supress in-exercise lactate, in agreement with previous work[154], but that this degree of ketosis likely sits in very close proximity to the lower threshold for this effect to be seen, potentially therefore manifesting under specific conditions.

The glucose lowering effect of exogenous ketosis is well documented when the KME is ingested alone[23,107,162], alongside carbohydrate and/or protein[23,37,151,160,170], and during 10 mM hyperglycaemic clamped conditions[168]. Here an average (-10 min onwards) lowering of 1.1 mM was found in High-KME compared to PLA alongside a 17.1% reduction in glucose-AUC, with Low-KME conversely not exhibiting any effect in accordance with previous work[154]. Howard et al.[150] observed glucose lowering at 1-2 mM βHB during treadmill exercise and established, via a primed 6,6-$^{2}H_{2}$-glucose infusion, that this was consequent of reduced hepatic output with no change to glucose clearance. Findings in my study are compatible with this dynamic as lowering wasn't seen across the cycle leg where the 75 g/hr exogenous carbohydrate provision likely supressed hepatic glycogenolysis and gluconeogenesis, thus glucose output[138,183,242], diminishing the scope for further KB-mediated reductions. Lowering was seen, however, at rest where exercise was absent as a potent driver for glucose homeostasis[183], and at the 10 km timepoint where plasma glucose concentrations were approximately doubled from rest, likely resultant of increased hepatic output due to the elevated exercise intensity[96,257,362] as participants increased their pace towards at the end of the effort and with the majority of circulating glucose likely being endogenous in origin as the final carbohydrate drink had been consumed ~1 hr prior, providing broadened opportunity for lowering of this output by ketosis.

The exact mechanisms underpinning this reduction in circulating glucose are unclear, with insulinotropic effects of exogenous ketosis inconsistently found, especially in exercise[72,107,170,171]. Accordingly, the glucose dynamics between High-KME and PLA observed in my work occurred in the absence of any ketotic alterations to circulating insulin or glucagon levels, further supporting that glucose lowering is not consequent of increased disappearance[37,409]. Plasma total GLP-1 & total PYY concentrations were both lowered by ketosis, but are unlikely to have modulated glucose kinetics as they primarily act to do so via insulin and glucagon[162,410–412]. In fact, in isolation they might be expected to elevate blood glucose via depressed GLP-1 increasing the rate of gastric emptying[413] and lowered PYY reducing insulin sensitivity[410]. However, the lowering of these hormones indicates that KME ingestion may slow the transit of small intestinal luminal nutrients, resulting in reduced GLP-1/PYY secretion from distal L-cells[410,413]. If the KME does delay gastric emptying, this could in-part explain

the observed reductions in circulating glucose as the absorption of ingested carbohydrate might be delayed[23,414,415], though this effect is yet to be observed in humans[158,416].

In the absence of indications of increased glucose clearance under exogenous ketosis, reduced hepatic output appears to be the driver of lowered plasma levels here. The impact of ketosis on gluconeogenesis in this work is ambiguous, however. Metabolomics data did not reveal a lowering of circulating alanine under ketosis, as has been previously observed to supress gluconeogenesis[136]. In contrast glutamine, another gluconeogenic substrate[417] and the most abundant circulating free amino acid[418], was elevated proportional to βHB concentration in line with prior work[136,189]. Additionally, whilst ketolysis might act to increase the balance of glycogen synthesis to glycogenolysis in extra-hepatic tissue during exercise via deactivation of AMPK[171,419] and accumulation of glucose-6-phosphate, an allosteric activator of glycogen synthase[420], this mechanism is unlikely to influence glucose turnover in the liver due to low SCOT abundance[24]. A novel finding, though, was the strong relationship between ΔAUCs for glucose and lactate (R^2 = 0.651), despite neither being associated with βHB ΔAUC. It could be therefore be speculated that some of the reduction to hepatic glucose output under ketosis is driven by diminished availability of lactate for gluconeogenesis via the Cori cycle[421], which might merit direct examination with tracer-labelled lactate[422].

The observed increase in plasma glutamine proportional to plasma βHB during cycling exercise here is potentially driven by KB oxidation in muscle, where 90% of this amino acid is produced[417], providing acetyl-CoA to the citrate synthetase reaction, consuming oxaloacetate, and thereby diminishing the transamination of glutamate to aspartate, leaving greater glutamate available for synthesis into glutamine[423,424]. Additionally, glutamine is produced by glia, with ketosis favouring the release of glutamine from the CNS, exchanged across the blood-brain barrier for leucine[423], though leucine levels were not seen to reduce proportional to βHB in my work. Circulating histidine, an essential amino acid, was also positively associated with βHB levels at the end of the cycle leg. This is likely due to reduced extra-hepatic histidine breakdown, consequent of ketosis-driven lowered availability of pyruvate for transamination[107,189].

Lipid Metabolism

Exogenous ketosis has been reported to either not affect[237] or suppress[107,150,152] circulating free FA levels during exercise. In the present study, plasma NEFA levels were unaffected by either KME condition compared to PLA. Levels remained supressed (<0.5 mM) at all but the 10 km timepoint however, likely due to the exercise being <2 hr[144,240,425], undertaken at carbohydrate-reliant intensities (RER: 0.94-0.99)[96,205,258], and conducted postprandially with high carbohydrate provision throughout[227]. Thus with adipose lipolysis rates, and subsequent release of free FA into circulation, likely already low[258], the antilipolytic capacity of KBs was limited[105,194].

Whilst total plasma NEFA concentrations were not influenced by ketosis across the exercise protocol presented here, a shift in FA composition with increasing βHB concentration was noted, especially at the conclusion of the cycle leg (+60 min), with metabolomic data revealing increased proportions of MUFAs and SFAs, alongside reductions in PUFAs. In the apparent absence of changes to adipose lipolysis and with the composition of the pre-exercise breakfast consistent for all conditions, this proportional shift towards greater FA saturation is potentially indicative of elevated hepatic *de novo* lipogenesis (DNL)[426] where the primary end-product is considered to be palmitate and thus the lipids synthesised are principally highly saturated[324]. This could be driven directly by elevated AcAc availability as a lipogenic substrate[29,427,428] or indirectly by the 1,3-butanediol component of the KME stimulating DNL[123], and may have been potentiated by the high fructose load of the carbohydrate drinks[318,321]. The role of insulin, which upregulates DNL[302,318,426], also cannot be ruled out as, whilst systemic circulating levels were not influenced by ketosis, it is possible that both secretion[175] and first-pass hepatic clearance[429] were elevated to the same extent. This would allow for increased insulin signalling isolated within hepatic circulation. Greater insight into this could be gained through insulin secretion-clearance modelling via additional assessment of plasma C-peptide concentrations[429]. Whilst [βHB] did not appear to influence circulating lipoprotein nor triglyceride profiles, this does not discount DNL as the source of this FA composition shift as very low density lipoprotein (VLDL) levels would not necessarily concurrently be elevated[430]. Though a reduction in PUFA concentration under exogenous ketosis during exercise has been previously reported[150], my work is the first to observe elevations in MUFA and SFA, and the only study to explore these dynamics in the absence of exogenous ketosis

depressing total NEFA levels[297]. The interaction between DNL and KME remains speculative however, with the relationship examined, at rest, utilising a deuterium tracer[431,432] in *Chapter 5*.

In this work, circulating sphingomyelins were found to correlate with βHB concentration. Increases have been described consequent of a ketogenic diet[433,434], but never previously explored under exogenous ketosis. Whether this elevation persists with chronic KME intake might merit investigation, as sphingomyelin levels are an independent risk factor for coronary heart disease due to their ability to retain cholesterol in arterial walls and to inhibit circulating lipolytic enzymes such as lecithin:cholesterol acyl transferase[435,436].

3.4.4 Cardiorespiratory Measures

In the present work, although no clear condition-effect emerged across the exercise protocol, an elevated V_E at 60 min in High-KME compared to PLA suggested that ketoacidosis[230] might have been modulating ventilatory drive[153] via alterations to BF, though circulating acid-base balance was not assessed to confirm this. Elevated V_E could potentially increase the oxygen cost of breathing[282], but as neither HR nor gross economy/$\dot{V}O_2$ were affected by condition, this doesn't appear to have made a tangible physiological impact. Findings here add to an equivocal evidence base concerning the interactions between cardiorespiratory measures and exogenous ketosis[31], with these relationships explored in greater depth in *Chapter 4*.

3.4.5 Gastrointestinal Distress & Perceived Exertion

Concerns have been posited over potential gastrointestinal distress from KME ingestion impacting athletic performance[31,111]. Disturbances, especially upper abdominal symptoms[146,152,229], have been found during cycling[125,146,152,229], running[151], and at rest[20] in what appears to be a dose-dependent manner, but are not universally seen[107,154,157,159,230]. In work presented here though, using a validated questionnaire[23,146,151,154,159], no differences were observed between conditions, despite doses equivalent to those in High-KME having previously caused GI upset[146]. This may be explained by the <2 hr exercise

duration[146,159] and well-trained participants potentially having sufficient prior 'gut training'[415] to handle the concurrent high loads of KME and carbohydrate.

No differences in perceived exertion were seen between the three experimental conditions. In a fasted state it has been reported that the disconnect between ventilatory drive[153,230] and perceived exercise intensity[285] during exogenous ketosis might increase both *Breathlessness* and *Anxiety of Breathing*. As no such observations were made here, it may be that a postprandial state modifies or dampens these mechanisms.

3.4.6 Appetite

Whilst KME ingestion has been shown to acutely suppress appetite[146,157,162], this is equivocally observed[294] with no clear pattern for the influence of exogenous ketosis present here, at rest or in-exercise.

Plasma concentrations of anorexigenic hormones total PYY and total GLP-1 (an incretin) were observed to be supressed under High-KME compared to PLA, with GLP-1 levels previously seen to be reduced during exercise and at rest, and PYY levels lowered at rest, by the KME[146,162,170,294,412]. This is the first study, though, to report a lowering of PYY by exogenous ketosis during exercise. Whilst these observations could be consequent of the KME slowing gastric transit, βHB is also known to antagonise GPR-41, a Gi/Go protein-coupled receptor expressed throughout the small intestines and believed to be responsible for gut-stimulated GLP-1 secretion[190]. The interaction between KBs and PYY, however, isn't well understood[162,410].

KME ingestion has been seen to blunt the rise in GDF-15, also an appetite suppressant[296], present across three weeks of cycling overtraining[193]. Though GDF-15 was elevated by exercise here as expected[295], no acute effects of ketosis were seen, as previously described[437], potentially because neither insulin nor glucagon were affected by exogenous ketosis[438].

Total ghrelin, the 'hunger hormone'[439] which activates neuropeptide Y neurons in the arcuate nucleus of the hypothalamus[439,440], was not lowered by High-KME compared to PLA. This sits in contrast to previous work that has robustly observed its supression[146,162,294,412,441], though a weak trend for condition

($p = 0.098$) was present so this study may have been underpowered to see an effect in this setting. This finding may have also been because ghrelin levels were already dampened by exercise that was undertaken at higher intensities than in prior studies[146,411,442,443], or by the pre/in-exercise carbohydrate[439], leaving less scope for lowering by ketosis. Additionally, assessing acyl-ghrelin instead of total ghrelin may have garnered differing results[294,412], whilst evaluation of catecholamines would have provided additional insight as ghrelin's secretion is stimulated by the catecholamine-induced activation of β1-adrenergic receptors[444].

Hormonal profiles were only established here pre- and in-exercise. As the suppressive effects of exogenous ketosis on ghrelin[146,294,412] and GDF-15[146] have been observed post-exercise, exploration in this timeframe may have led to alternate outcomes for these, and other, appetite-related hormones in work presented here.

3.4.7 Strengths & Limitations

Methodologies in this chapter adhered to acute exercise nutrition research best practice principles[386,445]. This included participants being competitively trained in both cycling and running, pre-Experimental visit familiarisation to the equipment and to the TT protocol, and the primary outcome measure being a TT which was expected to exhibit greater reliability than a TTE (CV: TT, 1-2%; TTE, >13%[386,394,446]). Additionally, pre- and in-exercise nutrition was provided in-line with current recommendations[3,5] and double-blinding was effective. Performance being assessed as a 10 km running TT after a 1 hr cycling pre-load conferred high translatability of findings as this imitates the physiological demands seen in many du-/triathlon race scenarios, most closely non-drafting 'Olympic' distance triathlons and 'Standard'/'Middle' distance duathlons[447].

This study is not without limitations though. Data published subsequent to the execution of this work have indicated that any ergogenic effects of exogenous ketosis for endurance performance, if present at all, are generally smaller in magnitude[31–34] than the effect size[107] used for sample size calculations. Therefore, this work might have been underpowered for its primary outcome. However, to see a significant condition effect for 10 km TT time based on results here between High-KME and PLA, the

slowest and fastest conditions on average, an infeasible sample size of 1,050[380] would be required (Cohen's D[392]: 0.0865, 'small' effect size[448]; α: 0.05; β: 0.8), indicating that my findings are not resultant of a constrained participant pool.

Additionally, using a proportion of power at the heavy-severe intensity domain threshold, assessed as critical power or maximal lactate steady state, might have given a more metabolically and physiologically consistent participant-to-participant workload for the cycle leg[391,396,449,450]. However, this would have required additional baseline visits[451], potentially impacting recruitment, and 70% $\dot{V}O_{2peak}$ reflected the intensity that well trained du/triathletes rode at during a 40 km cycle/10 km run race simulation[447]. The prescribed cycle leg workload did also appear to be within the heavy intensity domain for all participants, as plasma lactate under PLA was ≥1 mM higher than at rest, thus exercise was not in the moderate domain[451], whilst both lactate levels and $\dot{V}O_2$ did not progressively rise across the hour, indicating a metabolic steady state[142] and therefore that intensities were not in the severe domain.

Finally, running performance was assessed on a motorised treadmill wherein, due to technical limitations, the speed was adjusted in 0.1 kph intervals by a blinded study investigator upon participant request. This contrasts with non-treadmill running where athletes more subtly and subconsciously alter their pace, therefore the protocol may not have been suitably sensitive to detect small but tangible performance fluctuations[446,452].

3.4.8 Conclusions

Multimodal exercise performance was assessed as a 10 km running TT, which followed a 1 hr 70% $\dot{V}O_{2peak}$ cycling pre-load. Based on observations here, supplementing optimal carbohydrate feeding with a KME drink did not affect performance, regardless of the dose used and plasma βHB concentrations achieved. βHB concentrations did, however, proportionally influence circulating metabolite and satiety hormone profiles.

Chapter 4 - The Effect of Exogenous Ketosis on Moderate Intensity Cycling Economy

4.1 Introduction

The three key determinants of endurance exercise performance have been classically defined as the rate of maximal oxygen utilisation ($\dot{V}O_{2max}$), the fraction of this $\dot{V}O_{2max}$ at which exercise is sustainable (critical power/maximal lactate steady state/equivalent[396]), and exercise efficiency[453,454]. Additionally, the concept of physiological resilience, how these metrics change with fatigue, has recently been proposed as a fourth determinant[455].

Exogenous ketosis has been suggested to improve exercise efficiency[59,94,107], most commonly being evaluated as economy due to ketolysis confounding the substrate oxidation calculations necessary for determining energy expenditure[69,257,456]. Previous work has garnered equivocal results, though, with limited studies reporting a positive impact[71,232], and most observing no clear effect[94,154,157,230,233,457,458], for ketone monoester (KME) compared to placebo (PLA) conditions. However, as existing work is heterogenous with regards to KME dosing regimens, participant populations examined, and exercise modalities[459], intensities, and durations employed[31], it is difficult at present to draw robust conclusions.

Within the limited available literature, improvements to efficiency or economy under exogenous ketosis have only been observed in the absence of concurrent carbohydrate ingestion. Dearlove et al.[71] demonstrated that cycling delta efficiency was improved by ~7% after KME ingestion compared to PLA (1.8% absolute increase), with athletes exercising in an overnight fasted state and without in-exercise carbohydrate provision. Brady & Egan[232] subsequently found that the KME alone, but not KME co-ingested with carbohydrate, improved postprandial running economy by 3.6-4.5% at ~60-80% $\dot{V}O_{2peak}$ compared to a carbohydrate-only PLA. The smallest worthwhile change posited for running economy in the well-trained distance runners who were studied is 2.6%[454], thus their outcomes represented a 'real world' relevant improvement to this key performance determinant.

Previous findings may be explained by carbohydrate, when abundant, outcompeting ketone bodies (KB) as an oxidative substrate, whereas when pyruvate availability is lessened, ketone oxidation appears to increase[223]. Thus, a low carbohydrate availability ('low-carbohydrate') metabolic state may be necessary for ketolysis to tangibly contribute to energy provision during exercise. In lieu of carbohydrate restriction or fasting, which are unlikely to reflect how athletes optimally train or compete[5], a low-carbohydrate condition can be achieved through prolonged exercise, wherein progressive catabolism of skeletal muscle (SM) and liver glycogen results in diminishing rates of carbohydrate oxidation. Pertinently, this shift is observed even alongside 'gold standard' prior and intra-exercise exogenous carbohydrate provision[134,144,201,240,242], and is therefore in many circumstances obligate. Fatty acid (FA) utilisation is elevated in turn to compensate[246,258], which worsens economy due to the inferior oxygen efficiency (P/O) of β-oxidation when compared to glucose[134,240,252,456]. Under exogenous ketosis, however, sustained reduced circulating FA availability may supress this rise in lipid utilisation[105,198,204,258,460]. If rates of ketolysis were to progressively increase across a prolonged exercise bout, 'unlocked' by the inverse decline in carbohydrate oxidation, and this ketone oxidation supplanted a proportion of the energy provision that would otherwise increasingly come from β-oxidation, the rate at which economy worsens over time might slow, given βHB oxidation's superior P/O[91]. Thus, exogenous ketosis may arrest the rate at which economy progressively worsens, improving its resilience.

As well as in-exercise carbohydrate availability potentially influencing ketolysis, the reciprocal impact of exogenous ketosis on glucose oxidation remains equivocal[31,179]. Early histological findings of glycogen sparing consequent of KME ingestion[107] have not been replicated in subsequent work with debate ongoing surrounding the validity of differing glycogen staining methodologies[146,150,179]. Instead, through use of stable isotope tracers, exogenous carbohydrate oxidation rates can be quantified[144,185,257], bypassing the limitations of calculating substrate oxidation with indirect calorimetry under exogenous ketosis and facilitating direct interrogation of this potentially interdependent ketone-carbohydrate relationship.

The aim of this study was therefore to explore whether KME ingestion might rescue the decline in economy expected to occur after 2-4 hr of moderate intensity cycling, even alongside gold standard[3,5]

carbohydrate provision, with the impact of exogenous ketosis on oxidation of this exogenous carbohydrate also assessed. Subsequent high intensity work capacity was to be additionally investigated.

4.2 Methods

4.2.1 Study Design

This was a double-blind, randomised, and counterbalanced, repeated measures study. Participants completed a Baseline testing visit followed by two Experimental testing visits (*Figure 4.1*).

During the Baseline visit, cycling powers aligning with participants' lactate threshold 1 (LT1) and ventilatory threshold 2 (VT2) were established, as well as their $\dot{V}O_{2peak}$.

During the Experimental visits, participants consumed drinks containing either the KME or a taste and volume matched PLA. Carbohydrate drinks were provisioned throughout. Under each of these KME or PLA conditions, participants completed 4 hr of cycling at ~90% of LT1 power followed by a time-to-exhaustion (TTE) trial at their VT2 power, as determined at the Baseline visit.

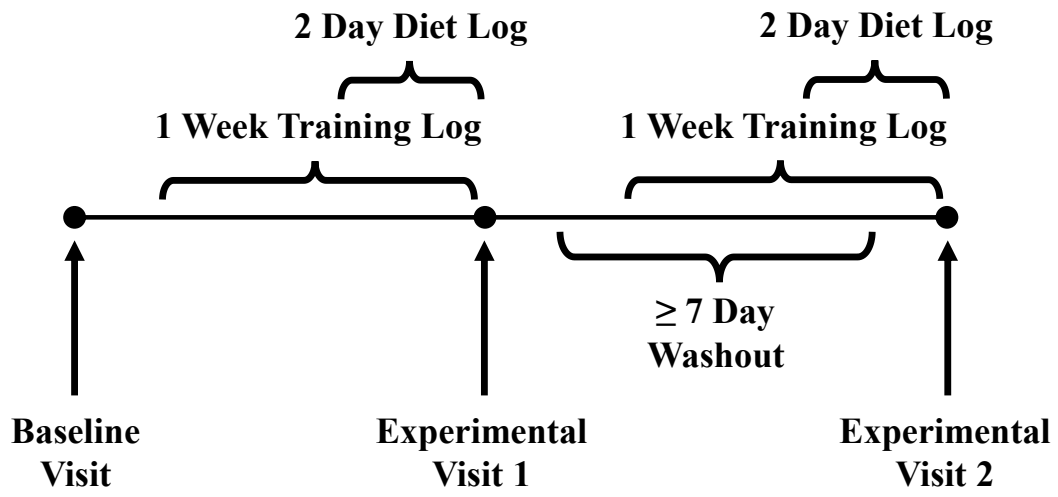


***Figure 4.1* - Overview of study timeline.**
Baseline and first Experimental visits separated by at least four days.

Participants

Ten participants were recruited from a pool of competitive cyclists in the Oxford area (*Figure 4.2*). Inclusion criteria are described in 2.2. Participant characteristics are summarised in *Table 4.1*, whilst characteristics for the five of these participants who were provisioned with ^{13}C (carbon-13) tracer

enriched drinks to ascertain exogenous carbohydrate, and plasma glucose, oxidation rates are summarised in *Table 4.2*. These five participants were the first five enrolled into the study.

A randomly generated matrix was used to ensure both condition orders were equally represented across the study to prevent order-effect biases, with participants assigned a predetermined visit order based on enrolment date.

Menstrual Cycle Control

Two of the five self-identified female participants were not on any form of contraceptive. Three self-reported as having an intrauterine device (IUD) fitted, two of which were hormonal in nature (both Levonorgestrel: one participant 13.5 mg, one 52 mg) and one as a copper oil. Details on how visit timings were controlled for menstrual cycle are detailed in 2.3.1.

***Table 4.1* - Participant characteristics (n = 10).**

		Male	*Female*
n		5	5
Age	yr	25.0 ± 1.3	24.8 ± 0.7
Height	cm	184.5 ± 3.2	172.1 ± 2.4
Weight	kg	79.2 ± 5.1	67.1 ± 4.5
Weekly End. Sport	hr	10.6 ± 1.7	11.4 ± 2.3
Weekly Cycling	hr	7.2 ± 0.8	7.0 ± 1.4
Performance Cycling	yr	5.4 ± 1.5	4.2 ± 1.3
LT1	W	256.0 ± 14.4	168.0 ± 12.4
VT1	W	281.0 ± 26.1	179.6 ± 9.4
	mL/min/kg	45.5 ± 1.2	33.2 ± 3.5
VT2	W	349.2 ± 15.1	244.6 ± 6.6
	mL/min/kg	53.0 ± 1.1	44.0 ± 3.5
$\dot{V}O_{2peak}$	W	452.0 ± 13.6	322.0 ± 12.0
	mL/min/kg	62.4 ± 1.5	51.0 ± 2.6

End, Endurance; LT1, Lactate Threshold 1; VT1, Ventilatory Threshold 1; VT2, Ventilatory Threshold 2. Data presented as Mean ± SEM.

***Table 4.2** - Participant characteristics (n = 5 who received ^{13}C enriched drinks).*

		Male	*Female*
n		3	2
Age	yr	23.7 ± 1.3	24.5 ± 0.5
Height	cm	180.5 ± 1.8	170.5 ± 7.0
Weight	kg	73.0 ± 3.7	68.9 ± 11.1
Weekly End. Sport	hr	9.3 ± 0.7	7.0 ± 3.0
Weekly Cycling	hr	8.0 ± 0.0	5.0 ± 1.0
Performance Cycling	yr	5.7 ± 2.2	3.5 ± 1.5
LT1	W	236.7 ± 3.3	155.0 ± 35.0
VT1	W	259.5 ± 24.5	172.5 ± 23.5
	mL/min/kg	46.5 ± 0.5	34.0 ± 1.0
VT2	W	336.7 ± 1.9	247.0 ± 18.0
	mL/min/kg	54.5 ± 1.5	46.0 ± 4.0
$\dot{V}O_{2peak}$	W	440.0 ± 11.5	320.0 ± 30.0
	mL/min/kg	64.0 ± 2.1	50.5 ± 3.5

Participants who were provisioned with ^{13}C enriched drinks to ascertain exogenous carbohydrate and plasma glucose oxidation. End, Endurance; LT1, Lactate Threshold 1; VT1, Ventilatory Threshold 1; VT2, Ventilatory Threshold 2. Data presented as Mean ± SEM.

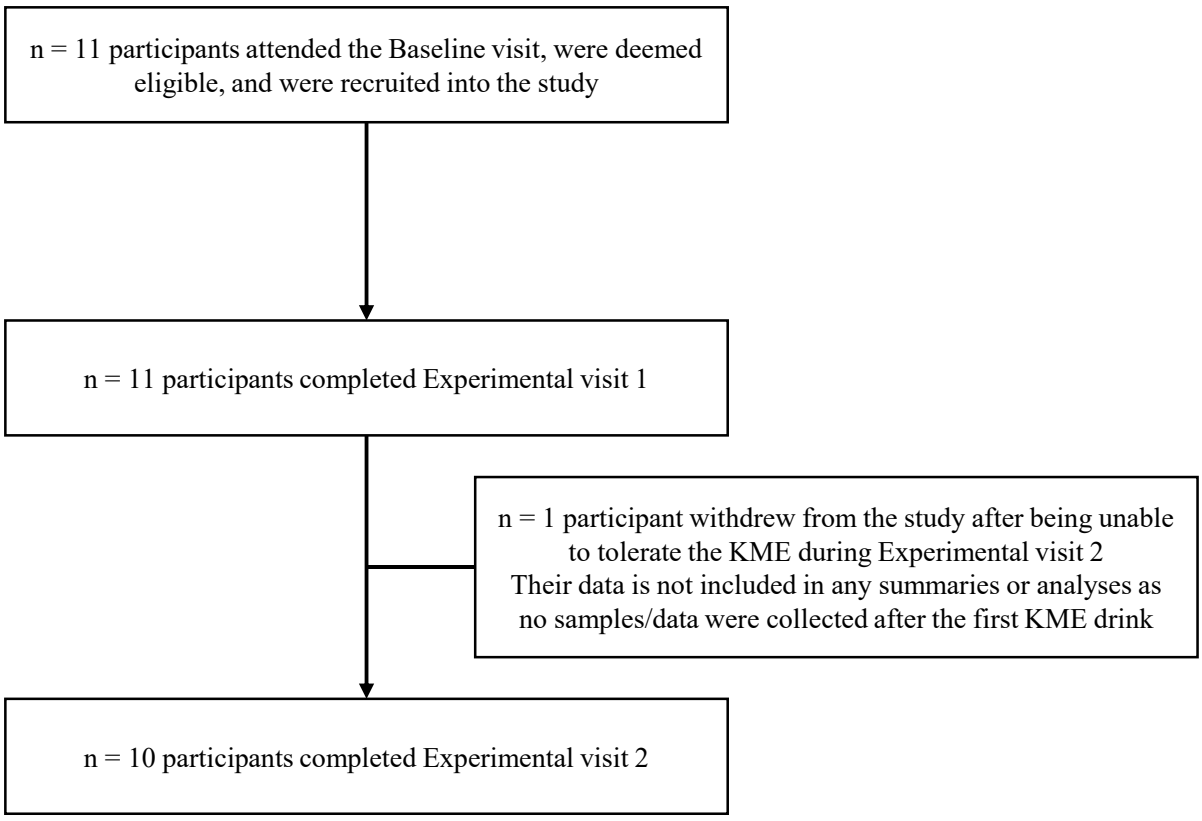


***Figure 4.2** - Study CONSORT diagram.*

4.2.2 Baseline Visit Testing

Participants were screened and enrolled into the study as per 2.3.2. They then completed two cycling step-tests on an ergometer (2.3.5) with cardiorespiratory measures monitored continuously (2.3.6).

Participants' power corresponding to lactate threshold 1 (LT1) was established during the first test. After completing a self-paced warm up followed by a 5 min break at rest, they commenced a step test (10 W increase every 4 min) starting at 190 W (male)/90 W (female). Blood lactate measures were taken in duplicate during the final 30 sec of each step using a handheld meter (Lactate Pro 2 Meter, Arkray Europe, High Wycombe, UK), with samples collected from the earlobe (28G Freestyle Lancet, Abbott, IL, USA). LT1 was taken as the highest power before a ≥0.5 mM rise in lactate[451] above baseline was observed. Baseline lactate was taken as the average across the resting and first power-step readings, as long as they did not differ by ≥0.2 mM, otherwise the first power-step value was used. Participants completed one further power-step after a ≥0.5 mM rise in lactate was observed to confirm that the rise was sustained. Participants then rested for 10 min before commencing the $\dot{V}O_{2peak}$ protocol.

To establish VT2 and $\dot{V}O_{2peak}$ (criteria: 2.3.6), participants cycled for 5 min at 50 W then immediately transitioned to a step test (20 W increase every 1 min) starting at 180 W (male)/90 W (female) until volitional exhaustion.

4.2.3 Experimental Visit Standardisation

Training Load & Dietary Control

Participants kept training load consistent for the six days prior to each visit and standardised their diet for the two days preceding each Experimental visit, both as described in 2.3.1.

The participants (n = 5; *Table 4.2*) who were given ^{13}C enriched in-exercise drinks were instructed to avoid naturally ^{13}C abundant foodstuffs for the four days preceding each Experimental visit (*Table 4.3*). All participants reported having undertaken at least one bout of ~exhaustive exercise in the first three of these days. Therefore, it was assumed that glycogen turnover in this healthy and active population would

be sufficient for ^{13}C to be depleted across the four days[461], and separate pre-Experimental visit glycogen depletion visits were not deemed necessary.

***Table 4.3* - List of foodstuffs known to be naturally highly enriched[352] in ^{13}C.**

Maize/corn	Fibrosol
Sugarcane	Polycose
Sorghum	Tomato Ketchup
Quorn	Worcestershire Sauce
Cornfed Chicken	Soy Sauce
Cornflakes	Bovril
Meats of North American origin (due to greater use of maize as feed)	Pineapple

4.2.4 Experimental Visits

Participants undertook two Experimental testing visits, with the protocols differing only in whether they consumed KME or PLA drinks. The protocol is described in *Figure 4.3*.

	FASTED	-2 hr	0 min	1 hr	2 hr	2 hr 15 min	2 hr 30 min	2 hr 45 min	3 hr	3 hr 30 min	4 hr	End TTE
Blood	X	*Std. Breakfast*	X	X	X	X	X	X	X	X	X	X
Blood Gas					X		X		X		X	X
Breath	X		X	X	X		X		X	X	X	
Indirect Calorimetry			X#	X*	X*		X#		X*	X#	X*	X$
RPE Scales			X	X	X		X		X	X	X	X
Appetite & GI Scales	X		X	X	X				X		X	
Urine					*Throughout*							

***Figure 4.3* - Experimental testing visit sampling timeline.**

*X, collected/measured at that timepoint. Duration of Indirect Calorimetry (respiratory gas exchange) data collection prior to the respective timepoint: #, 5 min; *, 20 min; $, throughout time-to-exhaustion (TTE) trial. GI, Gastrointestinal; RPE, Rating of Perceived Exertion; Std., Standardised.*

Upon arrival to the Clinical Research Unit (CRU), participants were cannulated and fasted blood samples obtained. Participants consumed a standardised breakfast then sat at rest for 2 hr.

At 2 hr post-breakfast, participants commenced the 4 hr cycle. This consisted of the same 1 hr workload protocols repeated four times uninterrupted, the first 40 min of which was spent at 90% LT1 power. For the final 20 min of each hour, a stepped protocol was undertaken to determine delta economy[250] with the power dropping to -60 W below 90% LT1 power, then sequentially rising to -45 W, -30 W, -15 W, +15 W (3 min per step) before returning to 90% LT1 for 5 min (*Figure 4.4*). Respiratory gas exchange (indirect calorimetry) data was collected across each of these 20 min periods and additionally for 5 min from 0 min, 2 hr 25 min, and 3 hr 25 min. Gross economy[456,462] was calculated during periods where the power was at 90% LT1 and respiratory gas exchange data was collected. Participants were instructed to keep cadence, a potential modifier of economy[252,456], consistent throughout the 4 hr cycle.

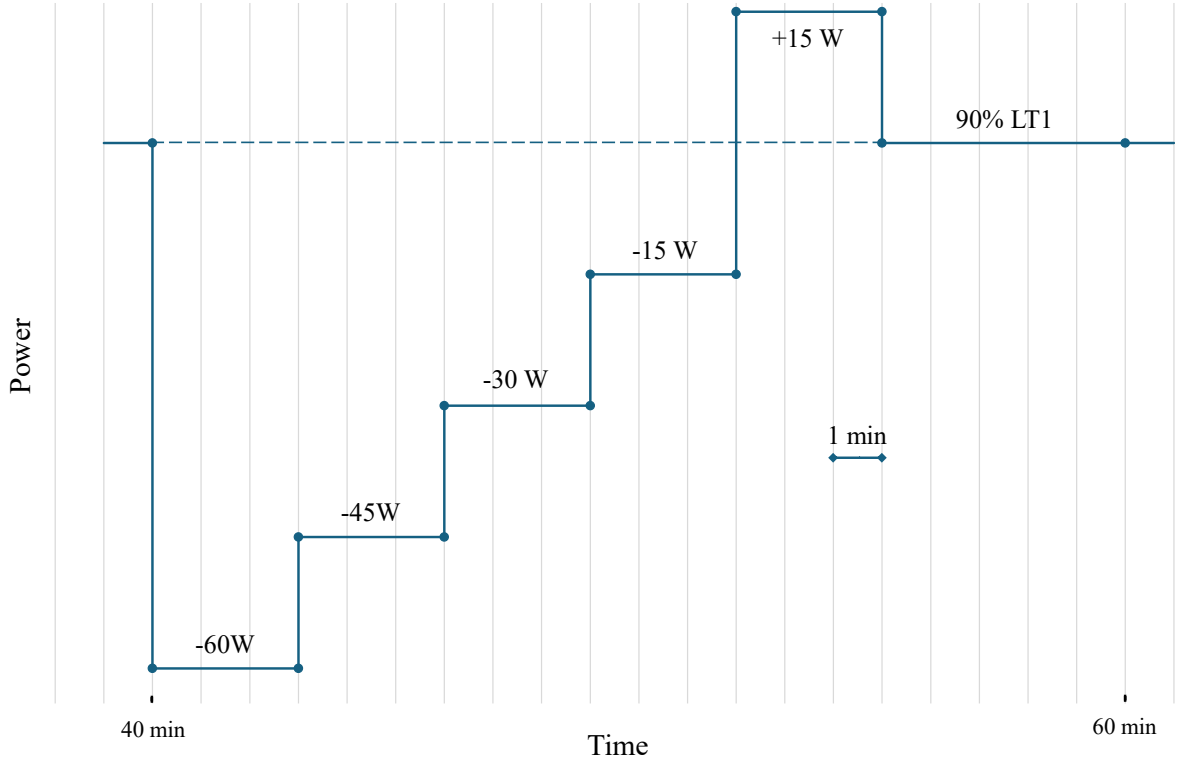


***Figure 4.4* - Illustration of power step protocol conducted over the final 20 min of each hour during the 4 hr cycle.**

LT1, Lactate Threshold 1 power. Changes in power (-60 W to + 15 W) are relative to 90% of LT1 power determined at the Baseline visit.

Participants were permitted to take breaks to visit the toilet whenever they chose, except for within 10 min prior to, and during, respiratory gas exchange data collection periods. The timings and durations of any breaks taken during the first Experimental visit were replicated exactly for the second visit. They were permitted to listen to music/watch entertainment throughout to alleviate boredom, though they were asked to keep the nature of this consistent between visits in case it might influence subjective measures.

After the 4 hr cycle, participants rested off the bike for exactly 10 min, before undertaking the TTE trial. Participants cycled for 2 min at 50 W then immediately transitioned to their non-fatigued VT2 power, which had been determined at the Baseline visit. Participants were instructed to give their 'best effort' and rode until they reached volitional exhaustion, or their cadence dropped <60 rpm for >10 sec. They were not permitted to listen to music during the TTE trial in case this might have influenced their motivation and so that they could always hear the study investigator. Respiratory gas exchange data was collected throughout with a blood sample taken immediately (in all instances ≤2 min) after exercise cessation. Participants were free to vary their cadence across the TTE effort. After a self-determined cool down, the visit concluded.

Standardised Breakfast

A standardised test breakfast was consumed 2 hr before commencing the 4 hr cycle, as per 2.3.4 (exact macronutrients: *Table 4.4*). It had low natural ^{13}C abundance[352] to minimise background enrichment.

***Table 4.4* - Standardised breakfast macronutrient composition.**

Total Calories	kcal	922	±	56
Carbohydrate	g	146.4	±	7.6
Protein	g	23.5	±	1.8
Fat	g	27.2	±	2.2

Data presented as Mean ± SEM.

Carbohydrate & KME/Placebo Drinks

Participants consumed 60 g/hr of carbohydrate during the 4 hr cycle as a 15% maltodextrin (Bulk, Colchester, UK) solution (*Table 4.5*). This was given in equal 20 g parts at 0, 20, and 35 min into each hour, with 35 min was used as participants were required to wear the respiratory gas exchange (indirect calorimetry) face mask from 40 min onwards. During endurance exercise of >2 hr, 60 g/hr is recommended to optimally aid performance[3,463] when supplementing with glucose/maltodextrin only.

A subset (n = 5) of participants' carbohydrate drinks were enriched with a U-^{13}C-glucose tracer (D-glucose U-$^{13}C_6$ 99%, #CLM-1396-0.5, Cambridge Isotope Labs, MA, USA) at a rate of 0.06 g/hr (ratio of 1 mg U-^{13}C-glucose per 1 g maltodextrin[144]).

***Table 4.5* - Composition of supplemental carbohydrate drinks.**

	Water (ml)	Maltodextrin (g)	U-^{13}C-Glucose (g)
Per 20 min	133.33	20	0.020
Per Hour	400	60	0.060
Per Four Hours	1600	240	0.240

15% carbohydrate drink compositions given in-exercise. Presented as amounts given per 20 min, per every 1 hr, and across the entire 4 hr cycle (n = 10). U-^{13}C-glucose given to n = 5 participants only.

KME/PLA drinks were given at 2 hr, 2 hr 35 min, and 3 hr 20 min (*Table 4.6*), diluted 2:1 with filtered water. This regime was chosen to ensure plasma βHB rose to >2 mM initially[107,110], with the subsequent drinks maintaining this elevation at a ~consistent concentration. The PLA solution was prepared and provided to participants as described in 2.3.3.

The KME/PLA were consumed from 2 hr onwards as it was during this period that a 'low-carbohydrate' state, where ketolytic rates might be elevated[223], was expected to start to be present[134,240]. Thus, the first 2 hr provided a glycogen depleting 'pre-load' that was identical between conditions.

***Table 4.6** - Timing and composition of KME and PLA drinks.*

Time		KME/PLA (mg/kg·BW)	KME/PLA (g)	KME-only (kcal)
Initial Drink	2 hr	600	43.9 ± 2.3	210.7 ± 10.9
Top Up Drink	2 hr 40 min	200	14.6 ± 0.8	70.2 ± 3.6
Top Up Drink	3 hr 20 min	200	14.6 ± 0.8	70.2 ± 3.6
Total		*1000*	*73.2 ± 3.8*	*351.2 ± 18.2*

KME/PLA drink compositions as amount per kg bodyweight (mg/kg·BW), as actual amount provided and as energetic value. Data presented as Mean ± SEM; n = 10.

No drinks were provided during the TTE as the respiratory gas exchange mask was worn throughout and any carbohydrate consumed would not be absorbed and oxidised in time to influence this effort (<20 min expected duration)[389,390].

4.2.5 Blood & Urine Sampling

Plasma Biochemistry

Venous blood samples (*Figure 4.3*) were collected as described in 2.3.4.

Plasma glucose, triacylglycerol (TAG), beta-hydroxybutyrate (βHB), non-esterified fatty acid (NEFA), lactate, glycerol, and urea concentrations were analysed (2.4) at all blood sampling timepoints. Total cholesterol, high density lipoprotein (HDL)-cholesterol, non-HDL-cholesterol, and Apolipoprotein B (ApoB) were analysed at the fasted timepoint.

Blood Gas, Electrolytes, & Haematology

At five of the blood sampling timepoints (*Figure 4.3*) an additional 2 ml of venous blood was drawn into a syringe (2 ml Emerald, BD, NJ, USA). To ensure blood gas levels reflected venous blood as closely as possible, this was taken immediately after blood was drawn for plasma. Samples were immediately analysed (always <3 min, per manufacturer's instructions) using a blood gas analyser (AN-500, i-STAT Alinity, Abbott, IL, USA; firmware version OSi20) with i-STAT EG7+ cartridges (#03P76-25, Abbott, IL, USA). From this, venous whole blood levels of the following were determined:

***Table 4.7* - Blood gas, electrolyte, & haematology measures.**

	Electrolytes (free ionized)	
Sodium (Na^+; mM)	Potassium (K^+; mM)	Calcium (Ca^{2+}; mM)
	Haematology	
	Haematocrit (Hct; % PCV [packed cell volume])	
	Blood Gas	
pH		Bicarbonate (HCO_3^-; mM)*
Partial pressure of CO_2 (pCO_2; kPa)		Partial pressure of O_2 (pO_2; kPa)
Base Excess (BE; ecf mM)*		Total CO_2 (TCO_2; mM)*

**, calculated from other measures. TCO_2, total CO_2 found in venous blood, in solution, bound to proteins, as bicarbonate [HCO_3], as carbonate [CO_3], and as carbonic acid [H_2CO_3]. Base Excess, the excess or deficit of base present in the blood with positive numbers indicating an excess of base and negative a deficit (i.e. excess of acid), standardised to haemoglobin of 5 g/dl to reflect the entire extracellular fluid (ecf).*

Measures that were calculated from directly determined metrics (BE/ HCO_3^-/ TCO_2) were recalculated manually to confirm that they had been correctly automatically determined. Equations used were as specified by the manufacturer (Art: 765860-01 Rev. E, Rev. Date: 29-Sept-2023; Abbott).

Urine

Urine was collected from when the first KME/PLA drink was given (2 hr) onwards. Total volume was recorded and a 2 ml sample collected for analysis of βHB and urea (2.4).

4.2.6 Cardiorespiratory Measures

Respiratory gas exchange (indirect calorimetry) data (*Figure 4.3*) was collected as per 2.3.6.

Correction of $\dot{V}CO_2$ for Ketoacidosis

The acidotic state of ketosis depletes bicarbonate stores which act to buffer this drop in pH[157]. This potentially inflates $\dot{V}CO_2$ with additional carbon appearance from non-oxidative sources. $\dot{V}CO_2$ resultant of ketoacidosis alone was therefore estimated when participants were in exogenous ketosis.

Total blood volume (TBV) was calculated as in 2.4. TBV was multiplied by the TCO_2 (mM) at a given timepoint to provide a total mmol value for CO_2 in the bloodstream. The change in this value from one timepoint to the next was determined (Δmmol), which was then divided by the duration between the timepoints to calculate the average change in CO_2 expired per minute (Δmmol/min). This was converted to L/min (24.466 L/mol) which was subtracted from total $\dot{V}CO_2$, providing a ketoacidosis-corrected $\dot{V}CO_2$ which was used in all subsequent calculations. This correction was not possible during the TTE as a metabolic steady state could not be assumed and blood gas measures were not assessed at the start of the effort.

4.2.7 Economy Measures

Gross economy was quantified during each 5 min 90% LT1 power period where respiratory gas exchange data was collected as the average $\dot{V}O_2$ across minutes 1-5 divided by the set-power:

***Equation 4.1* - Gross economy.**

$$\frac{\dot{V}O_2\ (L/min)}{\text{Work Rate (W)}} \quad (\text{Units: L/min/W})$$

Delta economy was calculated using an individual linear regression of the relationship between the average $\dot{V}O_2$ across each 3 min step, plus the 5 min 90% LT1, periods and their respective set-power, during the final 20 min of each hour during the 4 hr cycle (*Figure 4.4*). Where the R^2 of the regression line was <0.9, the datapoint which increased the R^2 value the most was removed, with only one datapoint ever being removed for a given regression equation. The slope of this relationship was taken as delta economy:

***Equation 4.2* - Delta economy.**

$$\frac{\Delta\ \dot{V}O_2\ (\text{L/min})}{\Delta\ \text{Work Rate (W)}} \quad (\text{Units: } \Delta[\text{L/min}]/\Delta\text{W})$$

4.2.8 Substrate Oxidation

Total carbohydrate and fat oxidation rates (g/hr), alongside energy expenditure (EE; kcal/hr), when not under a state of ketosis, were calculated from $\dot{V}O_2$ and $\dot{V}CO_2$ (both L/min) using the stoichiometric equations proposed by Jeukendrup and Wallis[257]. 'Low intensity exercise' equations were chosen as the 4 hr protocol was undertaken at an intensity <LT1 and <50% W_{max}:

***Equation 4.3* - Substrate oxidation during 'low intensity exercise'.**

$$CHO = \left(\left[4.344 \times \dot{V}_{CO_2}\right] - \left[3.061 \times \dot{V}_{O_2}\right]\right) \times 60$$

$$Fat = \left(\left[1.695 \times \dot{V}_{O_2}\right] - \left[1.701 \times \dot{V}_{CO_2}\right]\right) \times 60$$

CHO, carbohydrate.

Energy yield of each substrate was taken as 3.95 kcal/g for carbohydrate (assumption of 50% glucose, 50% glycogen contribution) and 9.75 kcal/g for fat[257]. Carbohydrate and fat oxidation rates were multiplied by their energy yields and these values added together to calculate EE.

Substrate oxidation calculations under exogenous ketosis were not possible[69] (2.3.6), with the exception of exogenous carbohydrate and plasma glucose oxidation as these were assessed by ^{13}C tracer methodologies. However exploratory analysis of this dataset was conducted to estimate these rates (4.2.9).

$^{13}C/^{12}C$ Enrichment Unit Conversion

^{13}C enrichment of CO_2 or carbohydrate that were quantified as δ‰ vs Vienna Pee Dee Belemnite (VPDB)[464] was converted to a tracer-to-tracee ratio (TTR; $^{13}C/^{12}C$) using[325]:

***Equation 4.4* - TTR.**

$$TTR = \left(\left[\frac{\delta‰}{1000}\right] + 1\right) \times 0.0112372$$

All ^{13}C enrichment data are presented as TTRs, with equivalent δ‰ vs VPDB values given where appropriate to facilitate comparison to other studies where this was the presented metric.

Carbohydrate Drink $^{13}C/^{12}C$ Enrichment

In-exercise carbohydrate drinks were enriched with 1 mg U-^{13}C-glucose powder (Cambridge Isotope Labs) per 1000 mg maltodextrin powder (Bulk), as in *Table 4.5*.

Maltodextrin carried a natural $^{13}C/^{12}C$ TTR of 0.011107833 ± 0.000000197 (-11.512 ± 0.018 δ‰ vs VPDB), with a range of 0.011107181 to 0.011108271 across 5 analyses. **This was assessed by Dr Christopher Day via elemental analysis isotope ratio mass spectrometry (EA-IRMS; EA: Isolink CN Elemental Analyser & Conflo IV, IRMS: Delta V Advantage; Thermo Fisher Scientific, Paisley, UK).**

Per information on the manufacturer's website, maltodextrin powder was assumed to be (as % total weight) 5% unbound water/non-caloric material, 8.1% simple sugars, and have an average dextrose equivalent (DE) of 19. Therefore the average degree of polymerisation (DP) was taken to be 6.316 glucose residue units per molecule (DE x DP = 120)[465,466]. As the manufacturer was not able to provide simple sugar composition, data from an equivalent product (GLUCIDEX IT 19, Roquette Frères, Lestrem, France) was used, with simple sugars assumed to be 2:5 glucose:maltose. Within the maltodextrin powder (excluding the 5% unbound water/non-caloric material), glucose and maltose were

assumed to be monohydrate. Maltodextrin polymers were assumed to carry one bound water molecule per every two anhydroglucose residue unit[467].

U-^{13}C-glucose was 99% enriched and therefore carried a TTR of 0.99.

U-^{13}C-glucose was assumed to be 2% non-caloric material and anhydrous, per the manufacturer's documentation.

Corrected for hydration, maltodextrin powder composition, $^{13}C/^{12}C$ ratios (when determining molecular mass), and purity, carbohydrate drinks carried a TTR of 0.012269339 ± 0.000000201 (91.850 ± 0.018 δ‰ vs VPDB).

Plasma Glucose $^{13}C/^{12}C$ Enrichment

Plasma glucose was extracted using methodology modified from Chacko et al.[468]. Plasma samples were vortexed and then centrifuged (2500 rpm, 4°C, 10 min) before 50 µL of supernatant was added to 500 µL of ice-cold acetone (A/0600/17, Fisher Scientific, Loughborough, UK). These samples were then vortexed and spun (2500 rpm, 4°C, 10 min) before either 25 µL or 50 µL of the deproteinated supernatant was transferred to a new tube and dried down under nitrogen in a water bath (15 min, 60 °C). Either 50 µL or 100 µL (if 25 µL or 50 µL supernatant, respectively, was used previously) of 2:1 acetic anhydride and pyridine (#320101 & #270970, Sigma-Aldrich, MO, USA) was then added, before samples were capped, vortexed, and heated in a water bath for 30 min at 60°C. Samples were then uncapped and dried down as before, before being reconstituted in 50 µL ethyl acetate (23882.296, Fisher Scientific, Loughborough, UK) and transferred to GC vials (Agilent, CA, USA) for analysis. $^{13}C/^{12}C$ glucose enrichment was to then be measured by gas chromatography-mass spectrometry (GC-MS; GC: 7890A, MS: 5975C, Agilent, CA, USA).

Though plasma glucose samples have been extracted, and an optimised GC-MS methodology has been established, enrichment data was unavailable in time for this thesis due to a GC-MS fault.

Expired CO_2 $^{13}C/^{12}C$ Enrichment

Breath samples were collected per *Figure 4.3* into evacuated 10 mL Exetainer tubes (Labco, High Wycombe, UK) using a clinically employed breath collection system (GaSampler™, QuinTron, WI, USA) wherein dead space gases were not collected. The collection bag was filled once with a breath sample then fully evacuated, before being filled again and sampled from to ensure there was minimal dilution with ambient air.

Breath samples were analysed using gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS; Finnigan GasBench II, Thermo Fisher Scientific). Carbon dioxide (CO_2) was separated from the presence of other gases through use of a 25m × 10µm × 0.32 mm capillary column (PoraPLOT Q, CP7551, Agilent). Samples were injected using the splitless injection mode with a 40µL injection volume at 110°C with the column helium flow kept at 1.2 mL/min. The sampling time was 10 min with the oven temperature at 35°C[325].

Exogenous Carbohydrate Oxidation

The 'Fasted' timepoint was taken as the background for each visit, as all carbohydrate oxidation would be endogenous in origin and therefore most closely reflect glycogen ^{13}C enrichment[185,469]. Calculations assumed that during exercise ^{13}C is not irreversibly lost in pools of TCA cycle intermediates and/or bicarbonate, and that $^{13}CO_2$ recovery in expired gases was complete or almost complete[470]. Exogenous carbohydrate (and plasma glucose) oxidation data were (/were to be) calculated from 1-4 hr to allow sufficient time for a steady state condition of ^{13}C turnover in the bicarbonate pool to be reached[471–473]. As the first KME/PLA drink was provided immediately after the 2 hr samples were collected, any data comparing conditions is presented from 2-4 hr. PLA data on its own is presented 1-4 hr to illustrate the full available time course.

Exogenous carbohydrate oxidation was calculated as:

***Equation 4.5* - Exogenous carbohydrate oxidation.**

$$\dot{V}_{CO_2} \times \left(\frac{R_{exp} - R_{ref}}{R_{exo} - R_{ref}}\right) \times \left(\frac{1}{k}\right)$$

$\dot{V}CO_2$, CO_2 production (L/min); R_{exp}, ^{13}C enrichment of expired (exp) CO_2; R_{exo}, ^{13}C enrichment of the exogenous (exo) ingested carbohydrate solution; R_{ref}, ^{13}C enrichment of expired CO_2 at baseline ('Fasted' timepoint taken as background); k, volume of CO_2 produced consequent of the complete oxidation of glucose (0.7467 L/g)[69].

Plasma Glucose Oxidation

Though the assessment of plasma glucose enrichment necessary for *Equation 4.6*, *Equation 4.8*, and *Equation 4.9* was not possible for this thesis, they are detailed below to demonstrate how this data would have been processed.

Based on the ^{13}C isotopic composition of plasma glucose (R_{glu}), oxidation of plasma glucose was calculated[469] as:

***Equation 4.6* - Plasma glucose oxidation.**

$$\dot{V}_{CO_2} \times \left(\frac{R_{exp} - R_{ref}}{R_{glu} - R_{ref}}\right) \times \left(\frac{1}{k}\right)$$

Endogenous carbohydrate (CHO) oxidation was taken as:

***Equation 4.7* - Endogenous carbohydrate oxidation.**

$$Endogenous\ CHO\ Oxidation = Total\ CHO\ Oxidation - Exogenous\ CHO\ Oxidation$$

Oxidation of muscle glycogen, either directly or through lactate shuttling[474] was calculated as:

***Equation 4.8* - Muscle glycogen oxidation.**

$$Muscle\ Glycogen\ Oxidation = Total\ CHO\ Oxidation - Plasma\ Glucose\ Oxidation$$

The amount of glucose released from the liver that was then oxidised was estimated[475] as:

***Equation 4.9* - Liver carbohydrate oxidation.**

$$Liver\ CHO\ Oxidation = Plasma\ Glucose\ Oxidation - Exogenous\ CHO\ Oxidation$$

Equation 4.7 and *Equation 4.8* were not calculable when under a state of exogenous ketosis as it was not possible to determine total CHO oxidation.

βHB Disposal Estimates

Total βHB disposal rates during KME condition visits were estimated as described in 2.4.

4.2.9 Exploratory Analysis - Gross & Delta Efficiency and Substrate Oxidation

Though calculations of substrate oxidation, and thus EE, from indirect calorimetry data are considered to be confounded by a state of exogenous ketosis (2.3.6), they have still been used in previous work employing the KME to ascertain cycling efficiency[31,71,94,476]. Thus, exploratory analysis was undertaken to facilitate direct comparison to this literature. Substrate oxidation and EE were calculated as in 4.2.8 under all conditions *including during exogenous ketosis*. $\dot{V}O_2$ and $\dot{V}CO_2$ data under KME were both:

1. corrected ('*ketone corrected*'), and
2. uncorrected ('*ketone uncorrected*')

for βHB oxidation to ascertain the magnitude of difference that said correction might make.

Only two studies have quantified βHB oxidation directly (with stable isotope tracers) in a human exercising model under exogenous ketosis[70,71]. The most applicable available dataset comes from the 50% W_{max}/'High Ketosis' condition found in Dearlove et al.[70] as 90% LT1 power in this study was on average 48.87% of W_{max}, and plasma [βHB] in 'High Ketosis' most closely matched those achieved here. Thus, a rate of 0.100 g/min was used for exploratory analyses. As 1 g βHB produces 0.8607 L CO_2

and consumes 0.9683 L O_2 when oxidised[69], 0.09683 L/min $\dot{V}O_2$ and 0.08607 L/min $\dot{V}CO_2$ were subtracted before substrate and EE calculations were made for the *ketone corrected* dataset. βHB oxidation[90] liberates 4.68 kcal/g, therefore 0.468 kcal/min was then added to the EE value.

From this, gross and delta efficiency was calculated, from both *ketone corrected* and *ketone uncorrected* EE calculations. Gross efficiency was quantified during each 5 min 90% LT1 period where indirect calorimetry was undertaken. It was calculated as the average EE divided by the 90% LT1 wattage:

***Equation 4.10* - Gross efficiency.**

$$\left(\frac{\text{EE (J/s)}}{\text{Work Rate (J/s)}}\right) \times 100 \quad \text{(Unit: \%)}$$

Delta efficiency was calculated using an individual linear regression of the relationship between EE during each 3 min step and the 5 min 90% LT1 period, and their respective wattage, during the final 20 min of each hour during the 4 hr cycle. As with delta economy, a maximum of one datapoint was removed to ensure $R^2 > 0.9$. Delta efficiency was taken as the reciprocal of the slope of the relationship (1/m)[71,94]:

***Equation 4.11* - Delta efficiency.**

$$1 \,/\, \left(\frac{\Delta\text{EE (J/s)}}{\Delta\text{Work Rate (J/s)}}\right) \times 100 \quad \text{(Unit: \%)}$$

In addition to determining EE as in 4.2.8 and above, EE was calculated using the following single equation proposed for exercise at this intensity[257] in order to compare values to previous work which had used this approach instead of calculating from individual substrate oxidation rates[70,71,94]:

***Equation 4.12* - Energy expenditure (single equation).**

$$\left(\left[0.575 \times \dot{V}_{CO_2}\right] + \left[4.435 \times \dot{V}_{O_2}\right]\right) \times 60$$

4.2.10 Subjective Measures

Generalised (Borg) and Localised Ratings of Perceived Exertion (RPE), Gastrointestinal (GI) Distress, and Appetite measures (*Figure 4.3*) were assessed as described in 2.3.4.

4.2.11 Blinding Efficacy

Participants were asked to try and identify each of the study conditions after they had completed all visits to assess drink-composition blinding effectiveness. They were requested to identify each visit based on 'the overall experience - incorporating drink taste, effort and GI perceptions during the 4 hr cycle and TTE'. They graded each selection with a certainty rating on a linear scale from 0 (completely uncertain) to 10 (completely certain).

4.2.12 Data Analysis

Outcome Measures

The primary outcome measure for this study was cycling economy (as gross and delta), whilst secondary outcomes were exogenous/plasma carbohydrate oxidation and TTE trial duration. In addition, measures of expired gas composition & ventilation (indirect calorimetry), HR, plasma biochemistry & blood gases, General and Localised RPE, Appetite, and GI distress were assessed.

Sample Size Calculation

A sample size calculation was performed in G*Power[380] with delta economy as the primary outcome measure. f = 0.459 was used based on Dearlove et al.[71] (Cohen's d[392] = 0.917; 'low-ketosis' vs 'control' conditions). With a significance level (α) of 0.05 and power (1-β) of 0.8 (2 conditions, 4 time-points), a

sample size of 6 was required. This was validated using f = 0.490 from Brady & Egan[232] (Hedges' g[477] = 0.98; running economy at 14 kph, the speed closest to LT1 based on graphically presented lactate data) which yielded n = 6 also. This study aimed to recruit 9-11 participants to account for attrition and overestimates of observed effects as data on KME supplementation in exercise economy/efficiency are equivocal[31,33,233] and Dearlove et al.[71] found their result through exploratory post-hoc analysis of a trend for an overall condition effect (p = 0.098).

Dataset Sample Sizes

One participant did not attempt the TTE trials through not wishing to risk the recurrence of an injury not related to the study. One participant immediately failed (cadence never >60 rpm) the TTE trial on both occasions due to self-reported exhaustion. Their elapsed time of 0 min is included in analysis as this still represents a performance outcome, though, as the test duration was insufficient to induce any tangible shifts in metabolism, no TTE-end blood samples were taken, and it was not possible to collect sufficient expired gas data over this period. Any TTE related measures therefore had in most instances a n-value 2 fewer than for the 4 hr phase of the study. Given this disparity, and as it represented an exercise bout distinct from the 4 hr protocol (heavy/severe vs moderate intensities and 10 min rest between protocols), TTE measures were analysed separately to the 4 hr protocol.

Plasma biochemistry is presented as n = 9 due to issues drawing blood from one participant. Blood gas, electrolytes, & haematology data carry a n = 8 due to this and a separate analyser failure. Fasted samples were analysed separately to confirm participants arrived at each visit in comparable metabolic states. Urine metabolite analysis carries a n = 5 due to participants being unable to urinate or forgetting to collect urine. Gas composition data from two participants was discarded as valid calibration could not be assumed after an equipment failure, thus all cardiorespiratory measures were n = 8. All n values are reported on applicable figures and tables.

Area-Under-The-Curve

Area-under-the-curve (AUC) was calculated across the 4 hr cycling protocol for selected metrics as per 2.5. Baseline for BE only was y = -8, the most negative datapoint, otherwise it was y = 0.

Statistics

Statistics were performed as described in 2.5. Simple linear regressions or Pearson's correlations were performed where appropriate between Δ*metric* values (the difference between KME and PLA values, as KME values *minus* PLA values) for two metrics.

4.3 Results

4.3.1 Plasma Biochemistry

Fasting

No differences between condition were found for any plasma metabolites when overnight fasted at the commencement of each visit (Appendix H).

4 hr Protocol

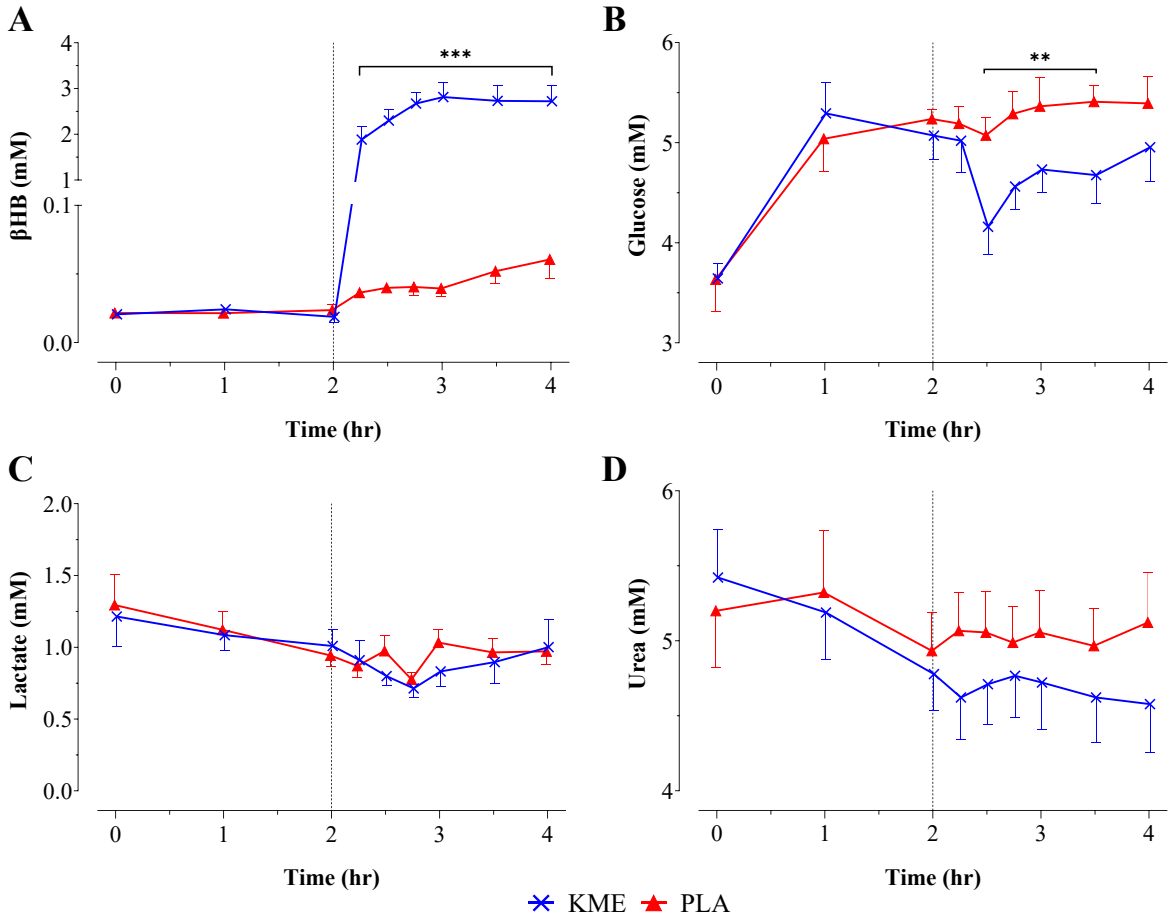


***Figure 4.5* - Plasma metabolites during the 4 hr cycle protocol under the KME & PLA conditions.** ***A**, βHB (mM); **B**, glucose (mM); **C**, lactate (mM); **D**, urea (mM) concentrations. Data presented as Mean ± SEM. Dashed line, first KME/PLA given immediately after 2 hr sample collected. ** $p < 0.01$, *** $p < 0.001$ between KME and PLA conditions at a given timepoint. $n = 9$.*

Ingestion of the KME drinks put participants into a state of exogenous ketosis, with sustained circulating βHB levels of ~2-3 mM achieved. Effects of time and condition, alongside a time-x-condition interaction, were found for plasma βHB levels ($p < 0.001$; *Figure 4.5-A*) during the 4 hr cycle protocol. At all timepoints after the first intervention drink, given immediately after the 2 hr sample was collected, levels were ~60-fold higher under the KME condition compared to PLA ($p < 0.001$), where levels remained low throughout. There was an effect of condition on βHB-AUC (KME: 4.862 ± 0.542, PLA: 0.132 ± 0.016 mM·hr; mean ± SEM; $p < 0.001$; *Figure 4.6-A*), indicating greater total βHB exposure under KME, though with substantial variance (min. AUC: 1.680, max. AUC: 6.539 mM·hr). ΔβHB-AUC (Δ represents KME-AUC *minus* PLA-AUC for a given metric) was not significantly predicted by participant age, height, weight, BMI, LT1 power, VT2 power, nor $\dot{V}O_{2peak}$ ($p \geq 0.308$).

Plasma glucose exhibited time, condition, and interaction effects ($p \leq 0.024$; *Figure 4.5-B*), though there was no difference between conditions for glucose-AUC (*Figure 4.6-B*).

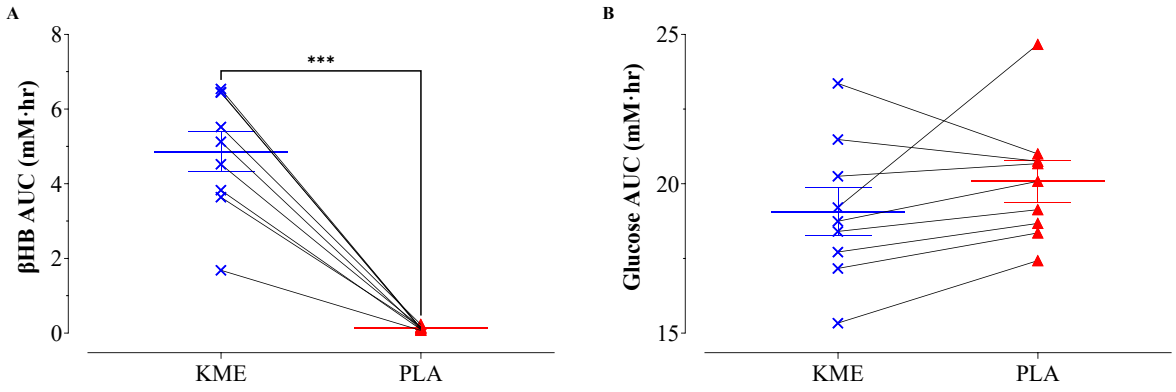


Figure 4.6 - Plasma metabolites AUC across the 4 hr cycle protocol under the KME & PLA conditions.

A, βHB (mM·hr); B, glucose (mM·). AUC, area-under-the-curve. Data presented as Mean ± SEM with individual values plotted. ********* *$p < 0.001$ between KME and PLA conditions. n = 9.*

There was a weak effect of time seen for plasma lactate ($p = 0.084$; *Figure 4.5-C*), whilst no condition or interaction effects were seen ($p \geq 0.384$). Lactate levels across the 4 hr were ~1 mM throughout, validating that participants remained under LT1 across the 4 hr protocol.

Plasma urea did not exhibit an effect of time (p = 0.157; *Figure 4.5-D*), though weak trends for both an effect of condition (p = 0.073) and an interaction effect (p = 0.095) were seen, indicating concentrations may have been lower under KME. There was no difference between conditions for urea-AUC (p = 0.137).

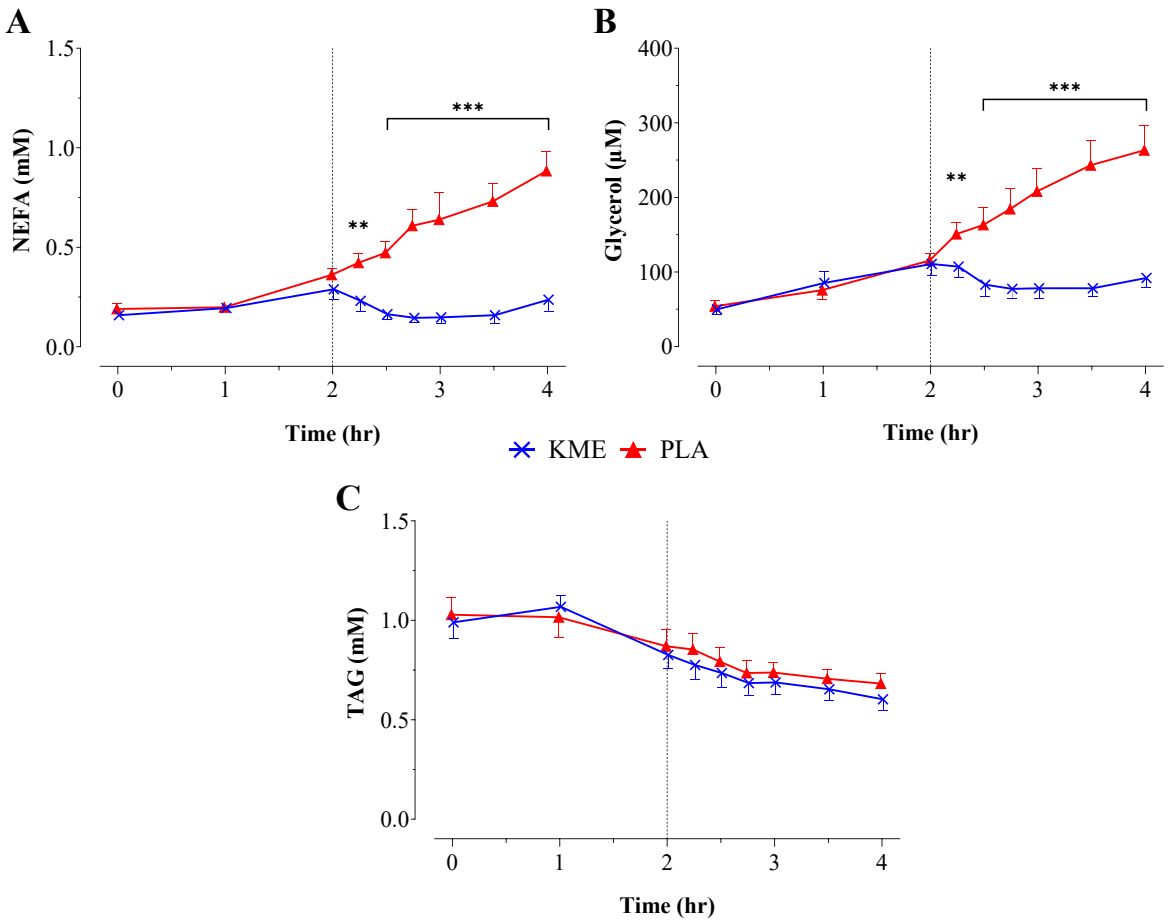


***Figure 4.7* - Plasma lipid metabolites during the 4 hr cycle protocol under the KME & PLA conditions.**

*A, NEFA (mM); B, glycerol (µM); C, TAG (mM) concentrations. Data presented as Mean ± SEM. Dashed line, first KME/PLA given immediately after 2 hr sample collected. ** p < 0.01, *** p < 0.001 between KME and PLA conditions at a given timepoint. n = 9.*

Plasma NEFA and glycerol concentrations both rose across the 4 hr protocol (p < 0.001; *Figure 4.7-A/B*). Condition and interaction effects were observed for both metabolites (p < 0.001), with levels supressed by KME. Both NEFA-AUC (KME: 0.784 ± 0.118, PLA: 1.720 ± 0.170 mM·hr; p < 0.001) and glycerol-AUC (KME: 338.2 ± 40.2, PLA: 564.8 ± 62.9 µM·hr; p < 0.001) were greater under PLA.

Though plasma TAG levels decreased over time ($p < 0.001$; *Figure 4.7-C*), neither a condition nor interaction effect were observed ($p \geq 0.493$). ΔβHB-AUC did not predict ΔAUCs for glucose, NEFA, or glycerol ($p \geq 0.253$).

TTE Completion

For samples taken at the point of exhaustion of the TTE trials (*Figure 4.8)*, plasma βHB was greater under KME compared to PLA ($p = 0.001$), whereas levels were lower for lactate, NEFA, and glycerol ($p \leq 0.049$). A trend for urea levels being lower under KME was seen ($p = 0.053$), with no between-condition differences observed for glucose or TAG ($p \geq 0.248$).

Plasma ΔβHB at the point of exhaustion significantly negatively predicted Δglycerol ($R^2 = 0.775$; $p = 0.009$) indicating greater elevations in βHB under KME compared to PLA predicted greater lowering of glycerol. Plasma ΔβHB did not predict any other metabolite ($p \geq 0.399$). Plasma Δglucose at the point of exhaustion was negatively associated with ΔTAG ($R^2 = 0.790$; $p = 0.007$) indicating that the greater the reduction in glucose under KME compared to PLA, the greater the increase in TAG.

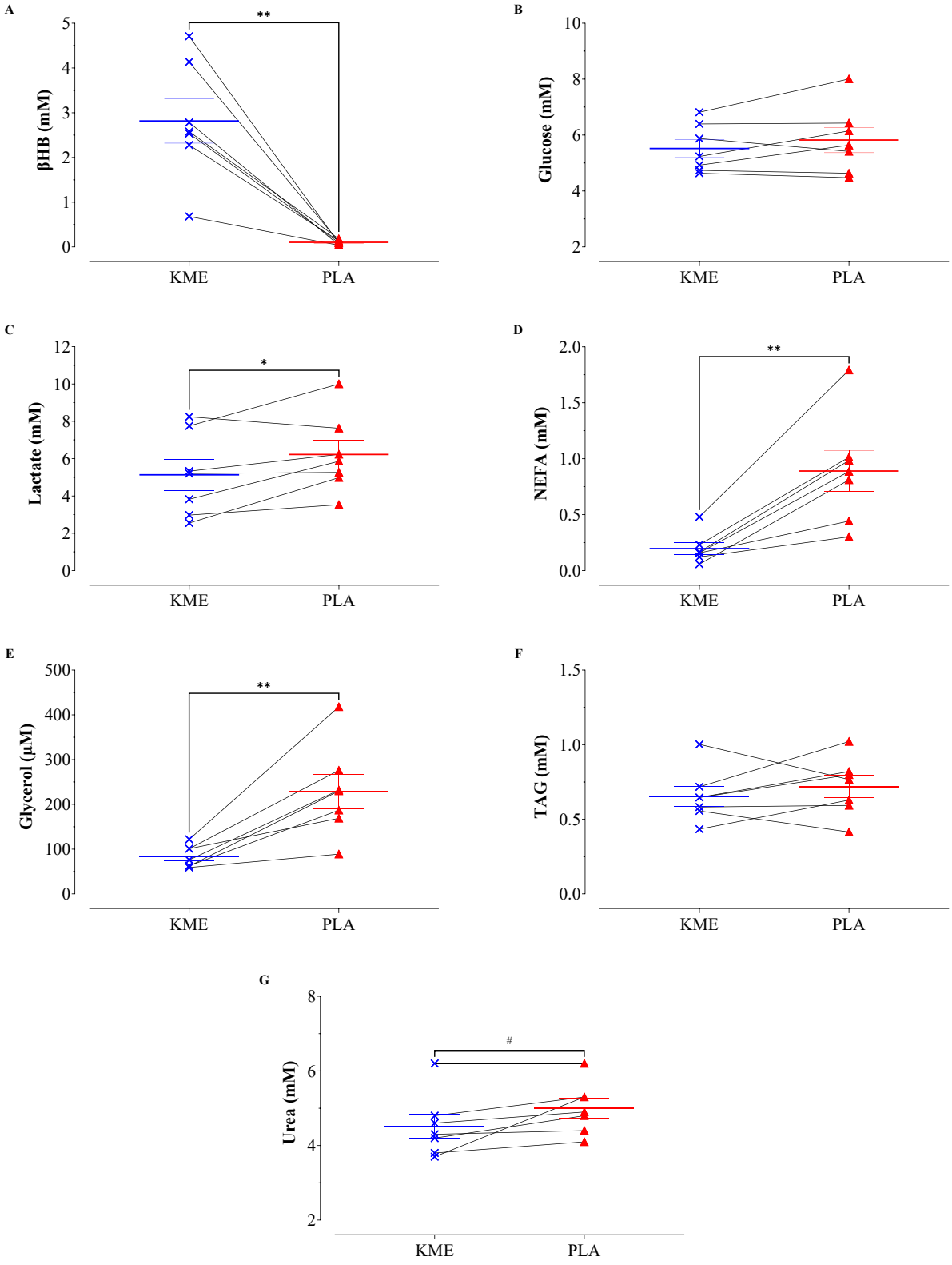


***Figure 4.8* - Plasma metabolites upon completion of the TTE trial under the KME & PLA conditions.** ***A**, βHB (µM); **B**, glucose (mM); **C**, lactate (mM); **D**, NEFA (mM); **E**, glycerol (µM); **F**, TAG (mM); **G**, urea (mM) concentrations. TTE, time-to-exhaustion. Data presented as Mean ± SEM with individual values plotted. # $p < 0.10$, * $p < 0.05$, ** $p < 0.01$ between KME and PLA conditions. $n = 7$.*

4.3.2 Cycling Economy

Economy

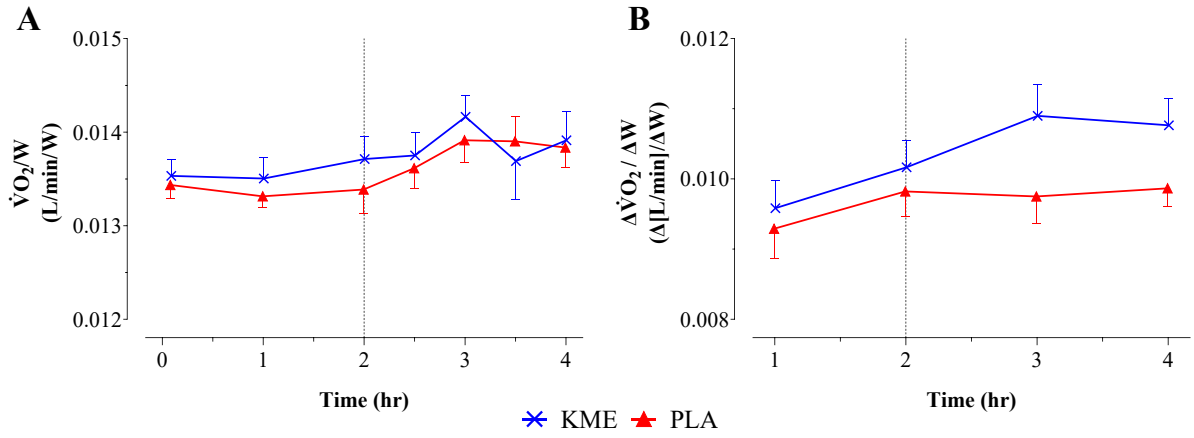


***Figure 4.9* - Cycling economy during the 4 hr cycle protocol under the KME & PLA conditions.**
***A**, Gross Economy (L/min/W); **B**, Delta Economy (Δ[L/min]/ΔW). Data presented as Mean ± SEM. Dashed line, first KME/PLA drink (given immediately after $\dot{V}O_2$ data had been collected for the 2 hr timepoint). n = 8.*

Gross economy tended to rise (worsen) over time ($\dot{V}O_2$/W; p = 0.061; *Figure 4.9-A*). No effect of condition (p = 0.255) nor an interaction effect (p = 0.487) were present.

Neither time (p = 0.153; *Figure 4.9-B*) nor interaction (p = 0.177) effects were observed for delta economy ($\Delta\dot{V}O_2/\Delta W$), however a condition effect was detected with economy greater (worse) under the KME condition compared to PLA (p = 0.027). Despite the lack of an interaction effect, exploratory post-hoc comparisons were conducted (not graphically presented in *Figure 4.9-B*) to ensure that this condition effect did not reflect baseline differences between conditions at 1 hr and 2 hr (i.e. prior to the first intervention drink) which could confound interpretation. These post-hoc analyses revealed that delta economy was greater at 3 hr (+11.78% compared to PLA; p = 0.019) and 4 hr (+9.11%; p = 0.007) under KME compared to PLA, but similar between conditions at 1 hr or 2 hr ($p \geq 0.198$). ΔDelta economy was not predicted at 3 hr ($p \geq 0.215$), nor at 4 hr ($p \geq 0.113$), by ΔBE, ΔTCO_2, ΔV_T, ΔV_E, plasma ΔβHB, Δglucose, ΔNEFA, venous ΔpH, or ΔHCO_3^-.

$\dot{V}O_2$ did not increase from 1-2 min to 2-3 min for any 3 min step, nor from 1-2 min to 4-5 min for any 5 min step ($p \geq 0.356$), with 0-1 min excluded in all instances (2.3.6), thus a steady state can be assumed at each datapoint. R^2 for linear regressions used to calculate delta economy were >0.9 in all instances.

When gross and delta economy were scaled by bodyweight, statistical findings were mirrored with regards to effects of time and condition, and interaction effects, as well as post-hoc relationships.

Cadence

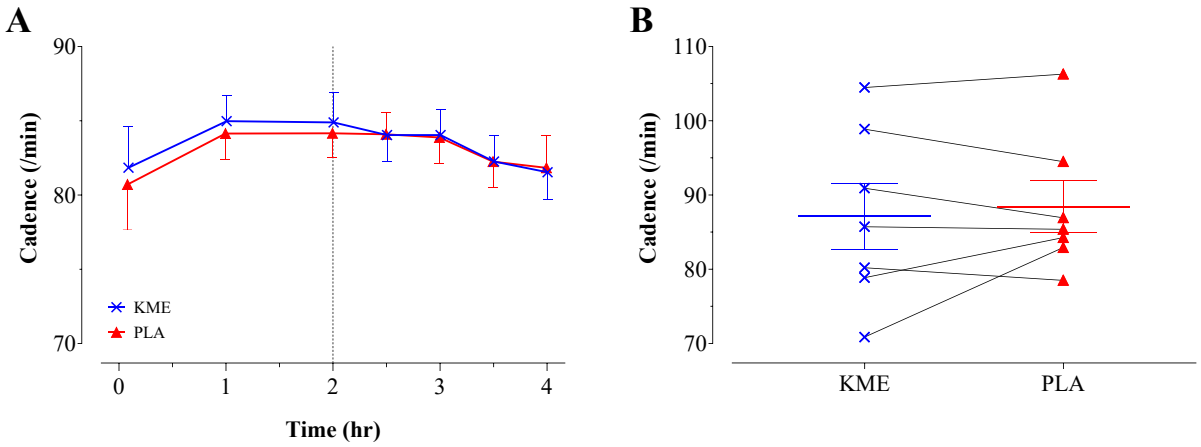


***Figure 4.10* - Cycling cadence under the KME & PLA conditions.**
A, *during the 4 hr cycle protocol;* ***B,*** *across the duration of the time-to-exhaustion (TTE) trial. Cadence, pedal revolutions per minute. Data presented as Mean ± SEM (with individual values plotted for B). Dashed line, first KME/PLA drink (given immediately after 2 hr timepoint). n = 10 (A); n = 7 (B).*

Cycling cadence did not exhibit time, condition, or interaction effects across the 4 hr protocol ($p \geq 0.219$; *Figure 4.10-A*), and did not vary by >10% between conditions for a given participant at any given timepoint. There was additionally no difference between conditions across the TTE trial ($p = 0.587$; *Figure 4.10-B*).

4.3.3 Time-to-Exhaustion

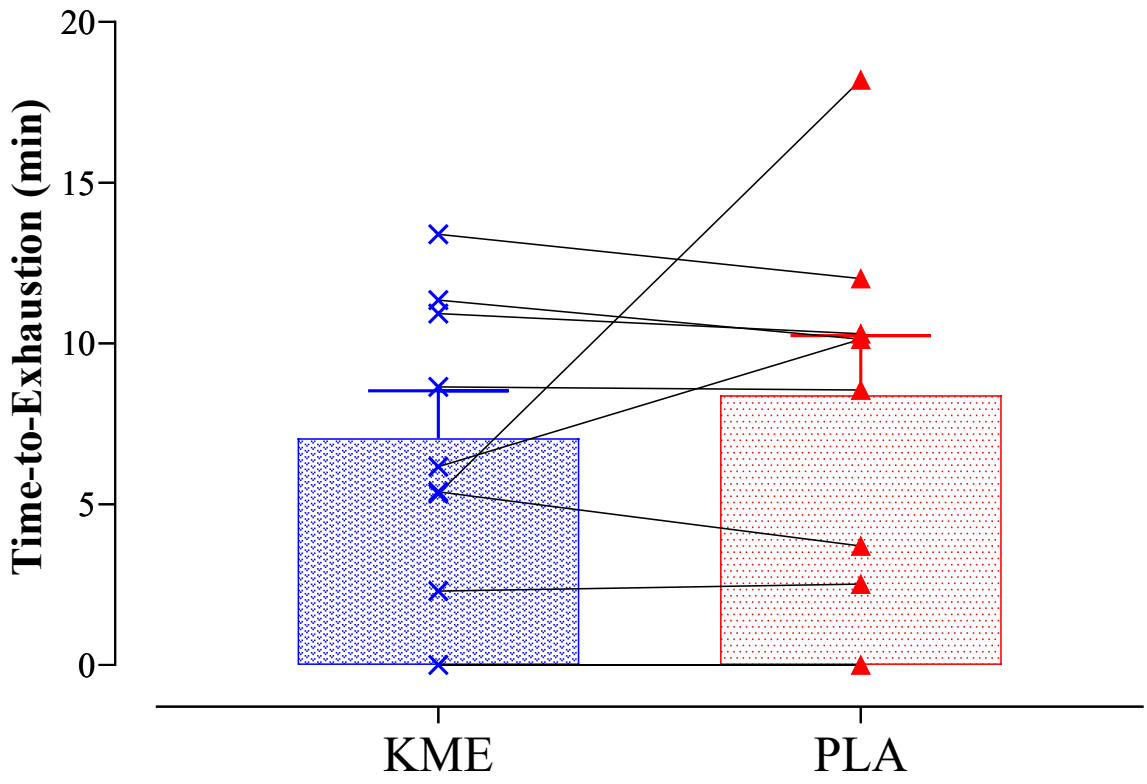


***Figure 4.11* - Effect of condition on TTE performance during the KME & PLA visits.**
TTE, time-to-exhaustion. Data presented as Mean ± SEM with individual values plotted. n = 9.

TTE during the trials at VT2 power proceeding the 4 hr protocol did not differ between conditions (KME: 7.06 ± 1.47, PLA: 8.39 ± 1.845 min; p = 0.498; *Figure 4.11*). One outlier was identified but re-analysis with this participant's data removed did not influence the outcome (KME: 7.27 ± 1.65, PLA: 7.17 ± 1.57 min; p = 0.874; n = 8).

Plasma ΔNEFA at the point of exhaustion positively predicted ΔTTE ($R^2 = 0.616$; p = 0.037), indicating that the greater the lowering of plasma NEFA under KME compared to PLA, the worse (shorter) TTE performance was. None of plasma ΔβHB, Δlactate, Δglycerol, ΔTAG, Δurea, Δglucose, venous ΔpH, ΔBE, ΔHCO_3^-, ΔHR, nor $\Delta \dot{V}CO_2$ otherwise predicted ΔTTE ($p \geq 0.171$).

No difference in TTE was observed between the first and second visits (first: 8.59 ± 1.91, second: 6.86 ± 1.36 min; p = 0.560), indicating that no order effect was present.

4.3.4 Substrate Oxidation

Substrate Oxidation Rates from Indirect Calorimetry (n = 8)

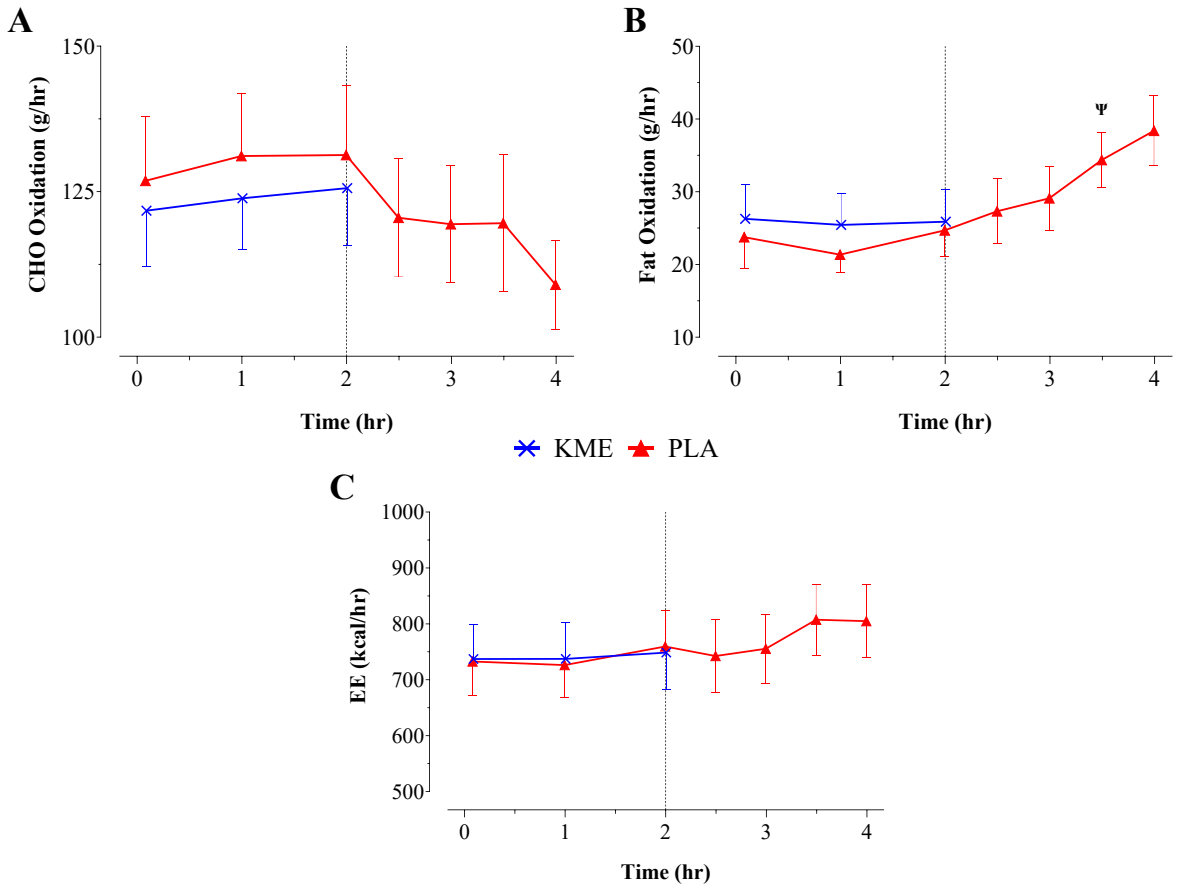


***Figure 4.12* - EE & substrate oxidation rates during the 4 hr cycle protocol under the KME (until 2 hr) and PLA (full 4 hr) conditions.**

A, Carbohydrate (CHO) Oxidation Rate (g/hr); B, Fat Oxidation Rate (g/hr); C, Total Energy Expenditure (EE; kcal/hr). Oxidation rates as grams per hour (g/hr). Data presented as Mean ± SEM. Dashed line, first KME/PLA drink (given immediately after 2 hr timepoint). $^{\Psi}$ *$p < 0.05$ between 1 hr and 3 hr 30 min for PLA. n = 8.*

For all participants, across the first 2 hr of the 4 hr protocol, there were no time, condition, or interaction effects for carbohydrate oxidation, fat oxidation, or overall EE ($p \geq 0.209$; *Figure 4.12*), indicating substrate metabolism didn't differ between conditions prior to the KME/PLA drinks.

Taking PLA condition data in isolation across the whole 4 hr period, no effects of time were found for carbohydrate oxidation or EE ($p \geq 0.111$). A significant effect of time ($p = 0.029$) was found for fat oxidation with rates rising over time.

Carbohydrate Oxidation from ^{13}C Tracer Methodologies (n = 5)

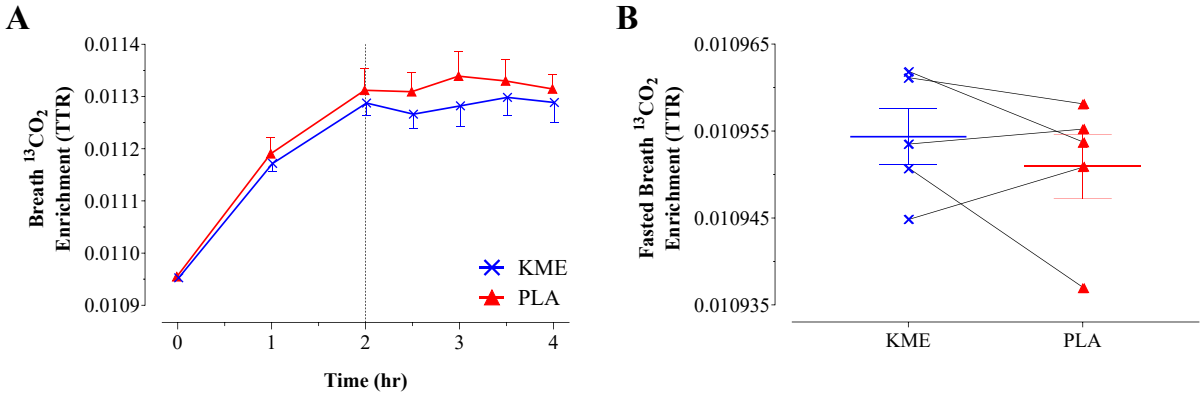


***Figure 4.13* - Expired $^{13}CO_2$ enrichment under the KME & PLA conditions.**

***A**, Across the 4 hr protocol as the tracer-to-tracee ratio (TTR; $^{13}C/^{12}C$); **B**, Fasting enrichment as the tracer-to-tracee ratio (TTR; $^{13}C/^{12}C$). 4 hr protocol, in-exercise ingestion of U-^{13}C-glucose (0.06 g/hr) and maltodextrin (60 g/hr). Data presented as Mean ± SEM (with individual values plotted for B). Dashed line, first KME/PLA drink (given immediately after 2 hr sample collected). n = 5.*

Expired CO_2 enrichment (TTR; $^{13}C/^{12}C$) increased over time ($p < 0.001$; *Figure 4.13-A*). Whilst an interaction effect was not present ($p = 0.176$), a weak trend for a condition effect was established ($p = 0.088$) indicating enrichment may have been lower under KME compared to PLA. Expired CO_2 enrichment did not differ between conditions when fasted upon arrival ($p = 0.382$; *Figure 4.13-B*), confirming that background enrichment was similar between conditions. It also did not differ at 0 hr ($p = 0.605$; postprandial and pre-carbohydrate drinks). Expired CO_2 enrichment as δ‰ vs VPDB can be found in Appendix I.

Exogenous carbohydrate oxidation exhibited an effect of condition with rates lower under KME than PLA (KME: 45.63 ± 0.78, PLA: 49.29 ± 0.86 g/hr; $p = 0.027$; *Figure 4.14-A*). However, no time or interaction effects were present ($p \geq 0.379$). As this work was potentially underpowered to establish an interaction effect when contrasted with existing literature[144,150,240,243,478–480], exploratory post-hoc analyses were undertaken (not graphically presented in *Figure 4.14-A*), revealing rates to be lower under KME at 2 hr 30 min ($p = 0.021$) and at 3 hr ($p = 0.006$). Rates at 2 hr, immediately pre-intervention, were not different between conditions ($p = 0.509$). Similarly, rates at 60 min, assessed separately, did not differ between condition ($p = 0.787$). Exogenous carbohydrate oxidation AUCs were found to be lower under KME (KME: 90.91 ± 4.04, PLA: 99.13 ± 4.50 g/hr·hr; $p = 0.021$; *Figure 4.14-B*).

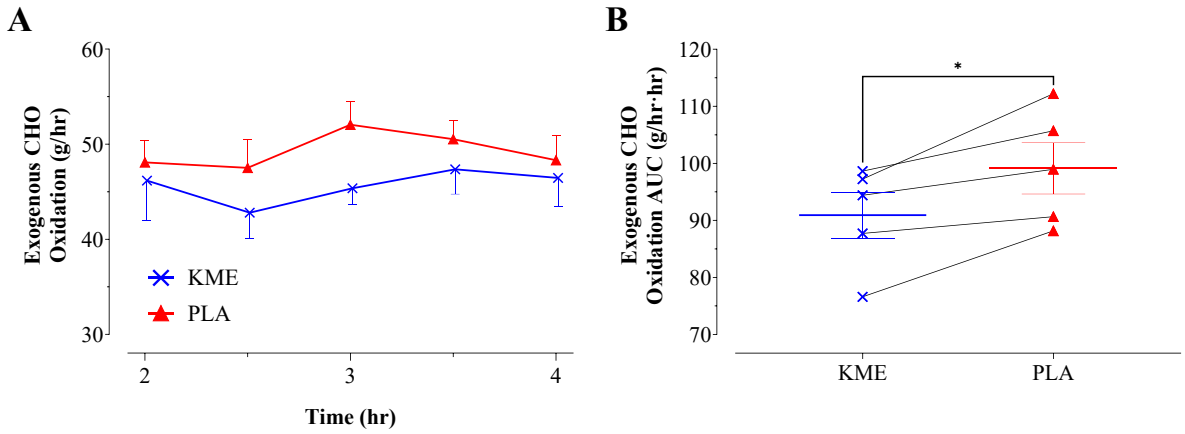


***Figure 4.14* - Exogenous carbohydrate oxidation rate from 2-4 hr of the 4 hr cycle protocol under the KME & PLA conditions.**

*A, Exogenous carbohydrate (CHO) oxidation rate (g/hr); **B**, Exogenous carbohydrate rate AUC (area-under-the-curve; g/hr·hr). In-exercise ingestion of U-^{13}C-glucose (0.06 g/hr) and maltodextrin (60 g/hr). First KME/PLA drink given immediately after 2 hr timepoint. Data presented as Mean ± SEM (with individual values plotted for B). * $p < 0.05$ between KME and PLA conditions. $n = 5$.*

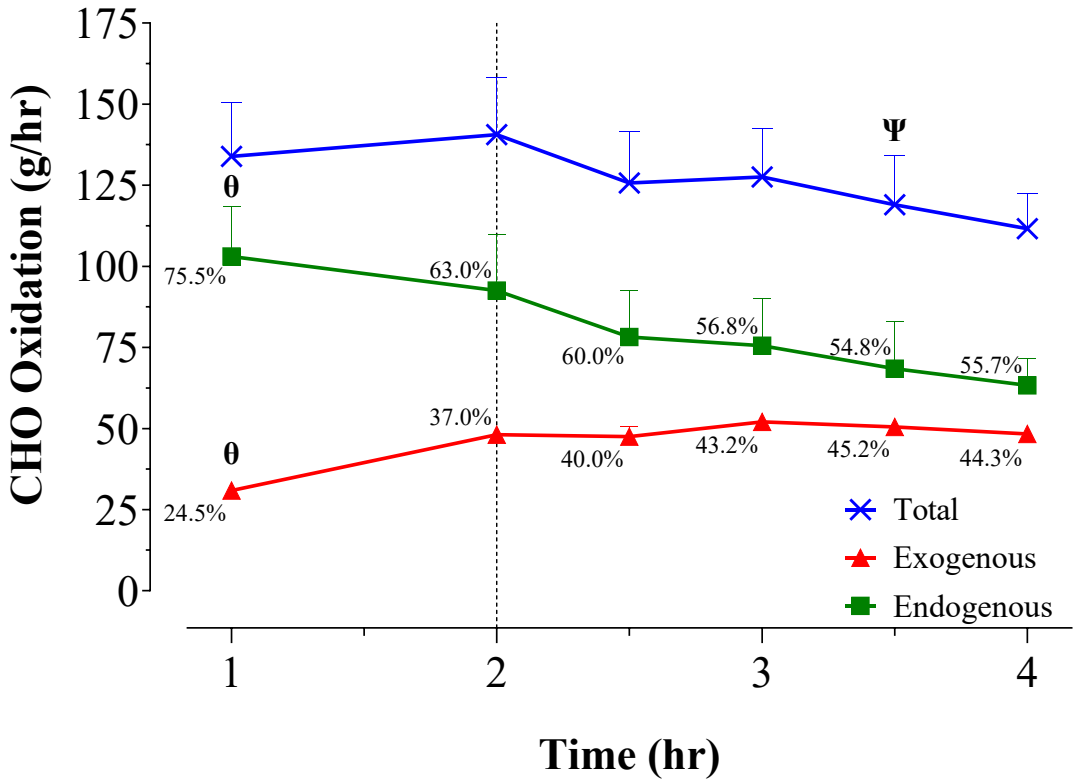


***Figure 4.15* - Total, exogenous, & endogenous carbohydrate oxidation rates from 1-4 hr of the 4 hr cycle protocol under the PLA condition.**

CHO, carbohydrate. In-exercise ingestion of U-^{13}C-glucose (0.06 g/hr) and maltodextrin (60 g/hr). Data presented as Mean ± SEM. Dashed line, first KME/PLA drink (given immediately after 2 hr sample collected). $^{\theta}$ $p < 0.05$ different to all other timepoints; $^{\Psi}$ $p < 0.05$ different to 3 hr. % values, proportion of total carbohydrate oxidation rate. $n = 5$.

For the 5 participants who consumed ^{13}C enriched carbohydrate in-exercise drinks, effects of time were observed for total, exogenous, and endogenous carbohydrate oxidation ($p \leq 0.043$; *Figure 4.15*) during the PLA condition, with total and endogenous oxidation rates falling, and exogenous oxidation rising, over time.

βHB Disposal Estimates

At an assumed 6 g/hr rate of βHB oxidation[71] and accounting for excretion into urine, 83.83 ± 0.70% of βHB disposal was unexplained across 2-4 hr of the KME visits, equating to 73.25 ± 4.57 g. If AcAc was assumed in circulation at 0.6 mM this explained only a further 0.38 ± 0.01% of disposal. Alternatively, an assumed 2.0 mM AcAc explained 1.27 ± 0.04%.

4.3.5 Cardiorespiratory Measures

4 hr Protocol

$\dot{V}O_2$ exhibited a time effect ($p = 0.041$; *Figure 4.16-A*) but not condition or interaction effects ($p \geq 0.202$). In contrast, condition ($p = 0.029$; *Figure 4.16-B*) and interaction ($p = 0.007$) effects were found for $\dot{V}CO_2$ in the absence of an effect of time ($p = 0.291$). A weak trend for significance was found for $\dot{V}CO_2$-AUC being greater under KME ($p = 0.078$). Significant interaction and condition effects ($p \leq 0.049$; *Figure 4.16-C*) were found for RER, in the absence of an effect of time ($p = 0.305$) or a difference between conditions for RER-AUC ($p = 0.322$). Statistical outcomes did not differ when $\dot{V}O_2$ and $\dot{V}CO_2$ were scaled by bodyweight.

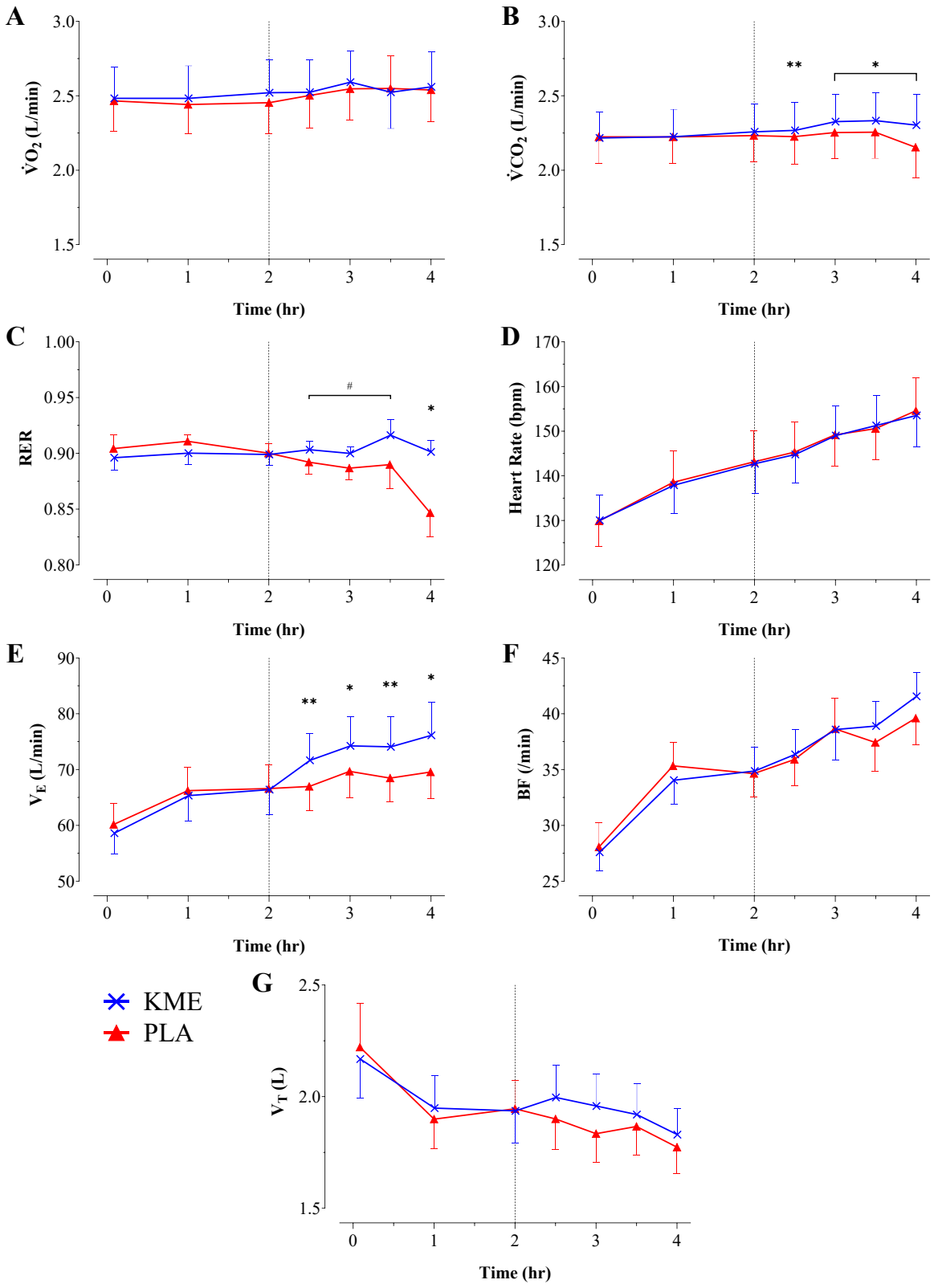


Figure 4.16 **- *Cardiorespiratory metrics during the 4 hr cycle protocol under the KME & PLA conditions.***

A, *Oxygen Uptake ($\dot{V}O_2$) in litres per minute (L/min);* ***B,*** *Carbon Dioxide Production ($\dot{V}CO_2$) in litres per minute (L/min);* ***C,*** *Respiratory Exchange Ratio (RER; $\dot{V}CO_2/\dot{V}O_2$);* ***D,*** *Heart Rate in beats per minute (bpm);* ***E,*** *Minute Ventilation (V_E) in litres per minute (L/min);* ***F,*** *Breathing Frequency (BF) in breaths per minute (/min);* ***G,*** *Tidal Volume (V_T) in litres (L). Data presented as Mean ± SEM. Dashed line, first KME/PLA drink (given immediately after 2 hr timepoint).* $^{\#}$ *$p < 0.10$,* * *$p < 0.05$,* ** *$p < 0.01$ between KME and PLA conditions at a given timepoint. $n = 8$.*

HR rose (*Figure 4.16-D*), and $\dot{V}O_2$/HR (data not presented) decreased, over time ($p < 0.001$) with no condition nor interaction effects found for either ($p \geq 0.139$). Significant effects of time were found for V_E, BF, and V_T ($p < 0.001$; *Figure 4.16-E/F/G*). There was a trend for an effect of condition for V_E ($p = 0.063$) alongside an interaction effect ($p = 0.003$). V_T exhibited a condition effect ($p \leq 0.014$) in the absence of an interaction effect ($p = 0.163$). Neither condition nor interaction effects were present for BF ($p \geq 0.364$). Though V_T-AUC was greater under KME compared to PLA ($p = 0.040$), no difference was found between conditions for V_E-AUC ($p = 0.144$).

There was a trend for ΔV_T-AUC and ΔV_E-AUC positively predicting each other's variances ($R^2 = 0.437$; $p = 0.053$). Otherwise, no cardiorespiratory ΔAUCs predicted each other variances, nor were predicted by ΔAUCs of any blood gas metrics ($p \geq 0.102$). ΔβHB-AUC did not predict $\Delta\dot{V}CO_2$-AUC, ΔV_E-AUC, ΔRER-AUC, nor ΔV_T-AUC ($p \geq 0.374$).

TTE Trial

Across the TTE trial (*Figure 4.17*), $\dot{V}CO_2$ and HR were higher under PLA compared to KME ($p \leq 0.026$), with no differences seen for any other metrics ($p \geq 0.100$).

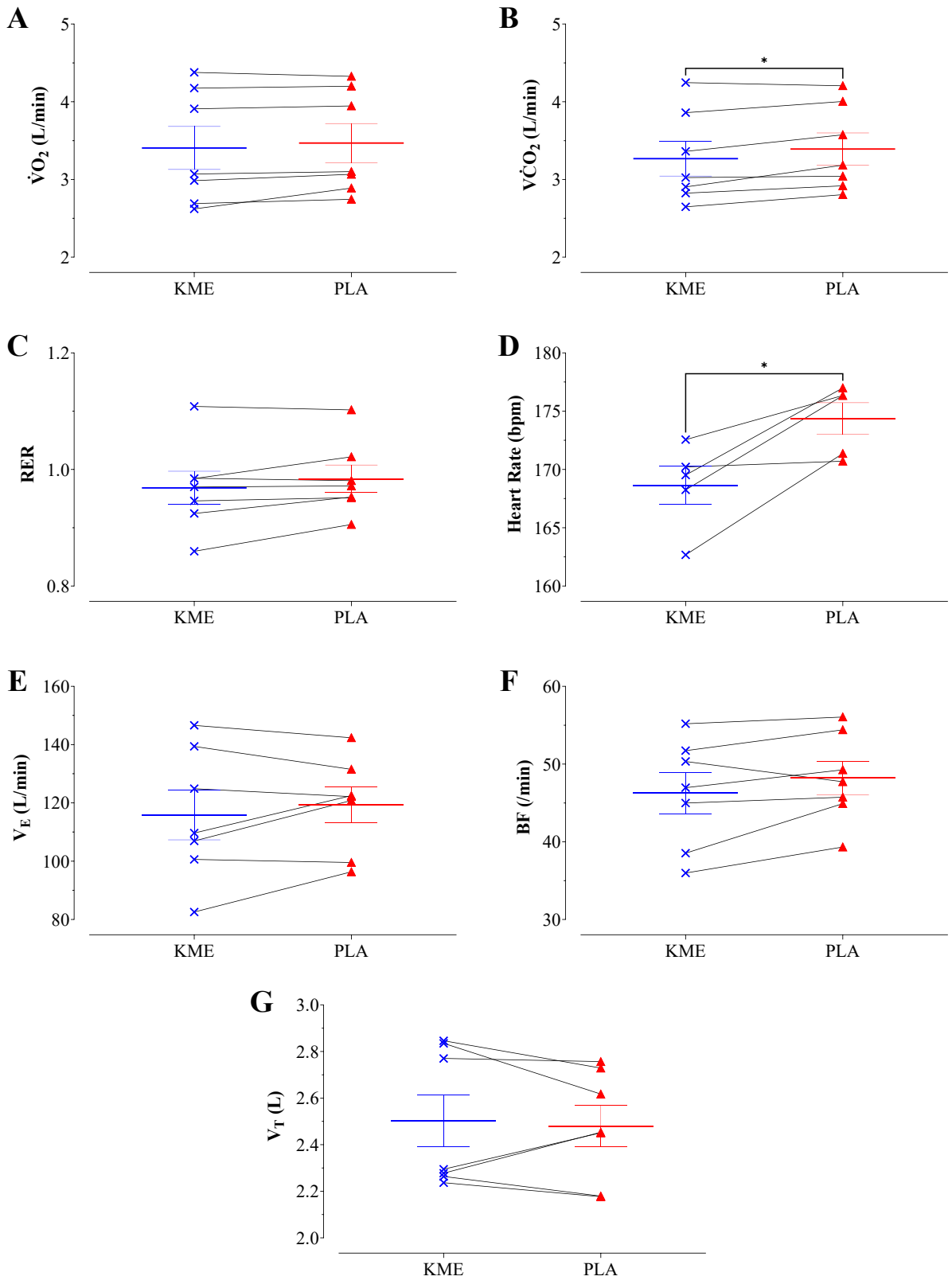


***Figure 4.17* - Cardiorespiratory & ventilatory metrics across the TTE trial under the KME & PLA conditions.**

A, *Oxygen Uptake ($\dot{V}O_2$) in litres per minute (L/min);* ***B,*** *Carbon Dioxide Production ($\dot{V}CO_2$) in litres per minute (L/min);* ***C,*** *Respiratory Exchange Ratio (RER; $\dot{V}CO_2/\dot{V}O_2$);* ***D,*** *Heart Rate in beats per minute (bpm);* ***E,*** *Minute Ventilation (V_E) in litres per minute (L/min);* ***F,*** *Breathing Frequency (BF) in breaths per minute (/min);* ***G,*** *Tidal Volume (V_T) in litres (L). TTE, time-to-exhaustion. Data presented as Mean ± SEM with individual values plotted.* ***** *$p < 0.05$ between KME and PLA conditions. n = 7 (A, B, C, E, F, G); n = 5 (D).*

4.3.6 Blood Gas, Electrolytes, & Haematology

4 hr Protocol

Venous whole blood pH exhibited condition and interaction ($p < 0.001$; *Figure 4.18-A*), but not time ($p = 0.825$), effects. A condition effect was found for HCO_3^- ($p = 0.004$; *Figure 4.18-B*), in the absence of time and interaction effects ($p \geq 0.293$). Time effects were established for pO_2 and pCO_2 ($p \leq 0.036$; *Figure 4.18-C/D*) without condition or interaction effects being observed ($p \geq 0.269$). BE and TCO_2 both exhibited time, condition, and interaction effects ($p \leq 0.003$; *Figure 4.18-E/F*). AUCs for pH, HCO_3^-, BE, and TCO_2 were all lower under KME compared to PLA ($p \leq 0.003$).

Plasma ΔβHB-AUC did not predict ΔAUCS for pH, HCO_3^-, or TCO_2 ($p \geq 0.115$), but did negatively predict ΔBE-AUC (*Figure 4.19*) indicating greater total βHB exposure under KME compared to PLA led to a more negative between-condition difference in BE-AUC.

No time, condition, or interaction effects were displayed for circulating sodium, potassium, calcium, or haematocrit ($p \geq 0.131$; Appendix J), except for a time effect being present for sodium ($p = 0.017$).

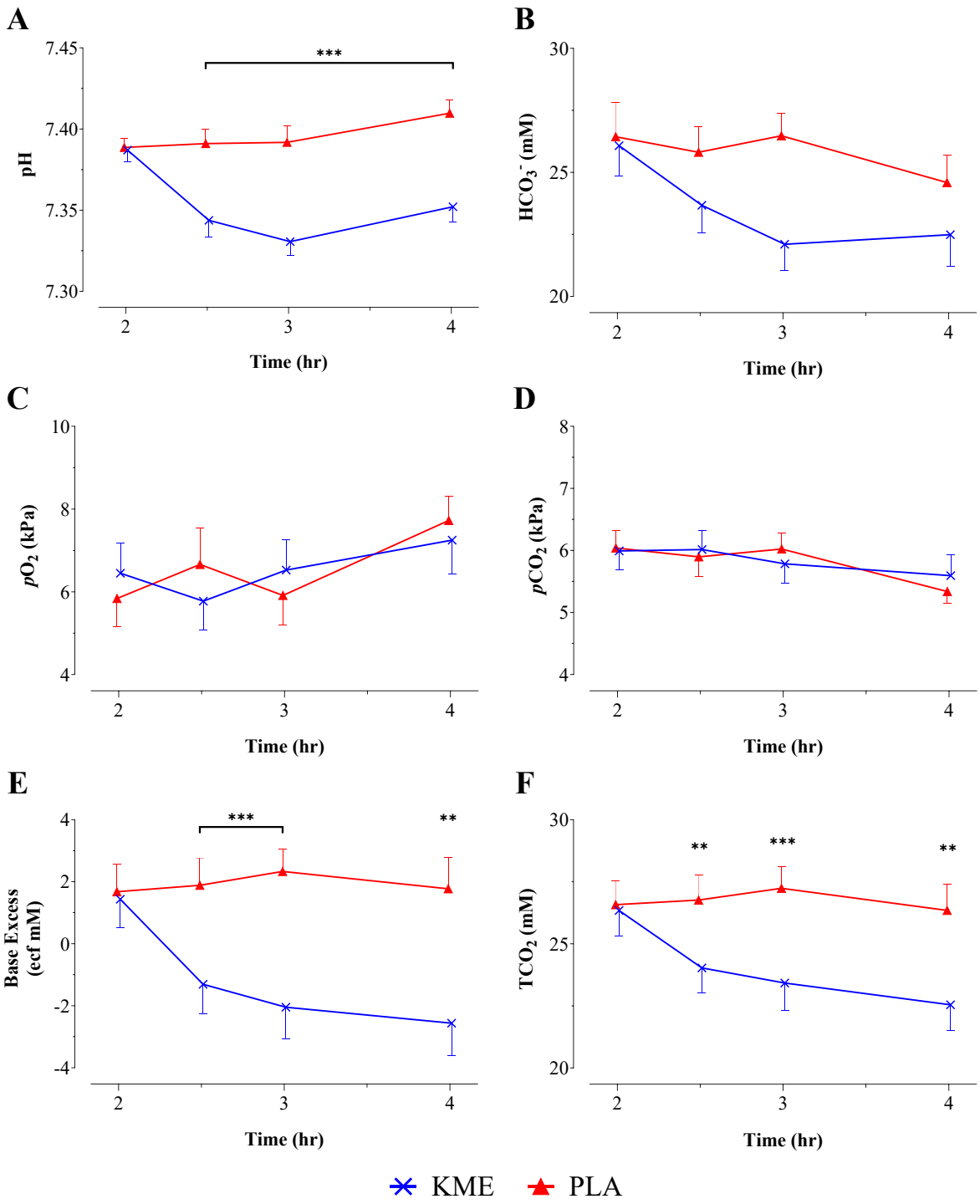


***Figure 4.18* - Venous whole blood gas measures during the 4 hr cycle protocol under the KME & PLA conditions.**

*A, pH; **B**, Bicarbonate (HCO_3^-; mM); **C**, Partial Pressure of Carbon Dioxide (pCO_2; kPa); **D**, Partial Pressure of Oxygen (pO_2; kPa); **E**, Base Excess (BE; ecf mM) **F**, Total CO_2 (TCO_2; mM). First KME/PLA drink given immediately after 2 hr timepoint. Data presented as Mean ± SEM. ** $p < 0.01$, *** $p < 0.001$ between KME and PLA conditions at a given timepoint. n = 8.*

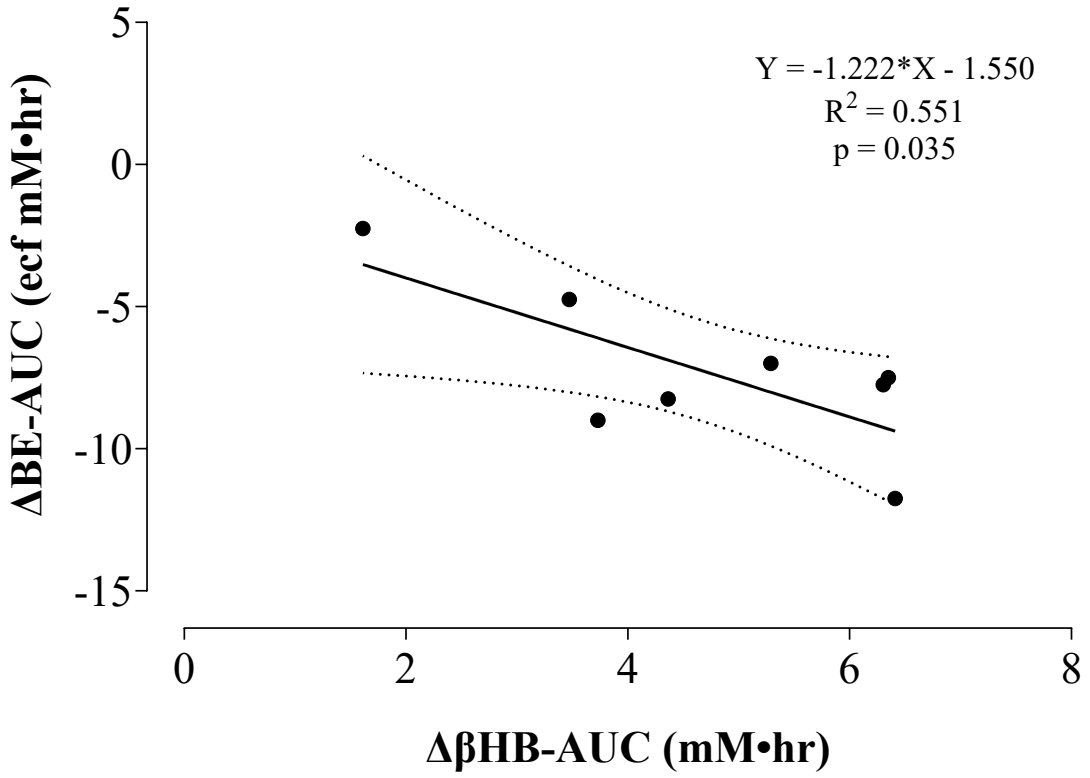


***Figure 4.19* - ΔβHB-AUC vs ΔBE-AUC across the 4 hr cycle protocol.**
AUCs as mM·hr. Δ, between condition difference calculated as KME minus PLA; AUC, area-under-curve; BE, Base Excess (ecf). AUC baselines: βHB - 0 mM, BE - -8. Solid line, linear best fit; dashed lines, 95% confidence intervals. n = 8.

TTE Completion

At completion of the TTE trial (point of exhaustion; *Figure 4.20*), venous blood pH and HCO_3^- were lower ($p \leq 0.037$) under KME compared to PLA, with a trend for seen for BE being lower ($p = 0.064$). No differences between conditions were found for pO_2, pCO_2, or TCO_2 ($p \geq 0.133$). There was a trend for plasma ΔβHB negatively predicting ΔBE ($R^2 = 0.643$; $p = 0.055$), though plasma ΔβHB did not predict ΔpH or ΔHCO_3^- ($p \geq 0.265$).

No effect of condition was found for venous sodium, potassium, calcium, or haematocrit upon completion of the TTE trial (Appendix J).

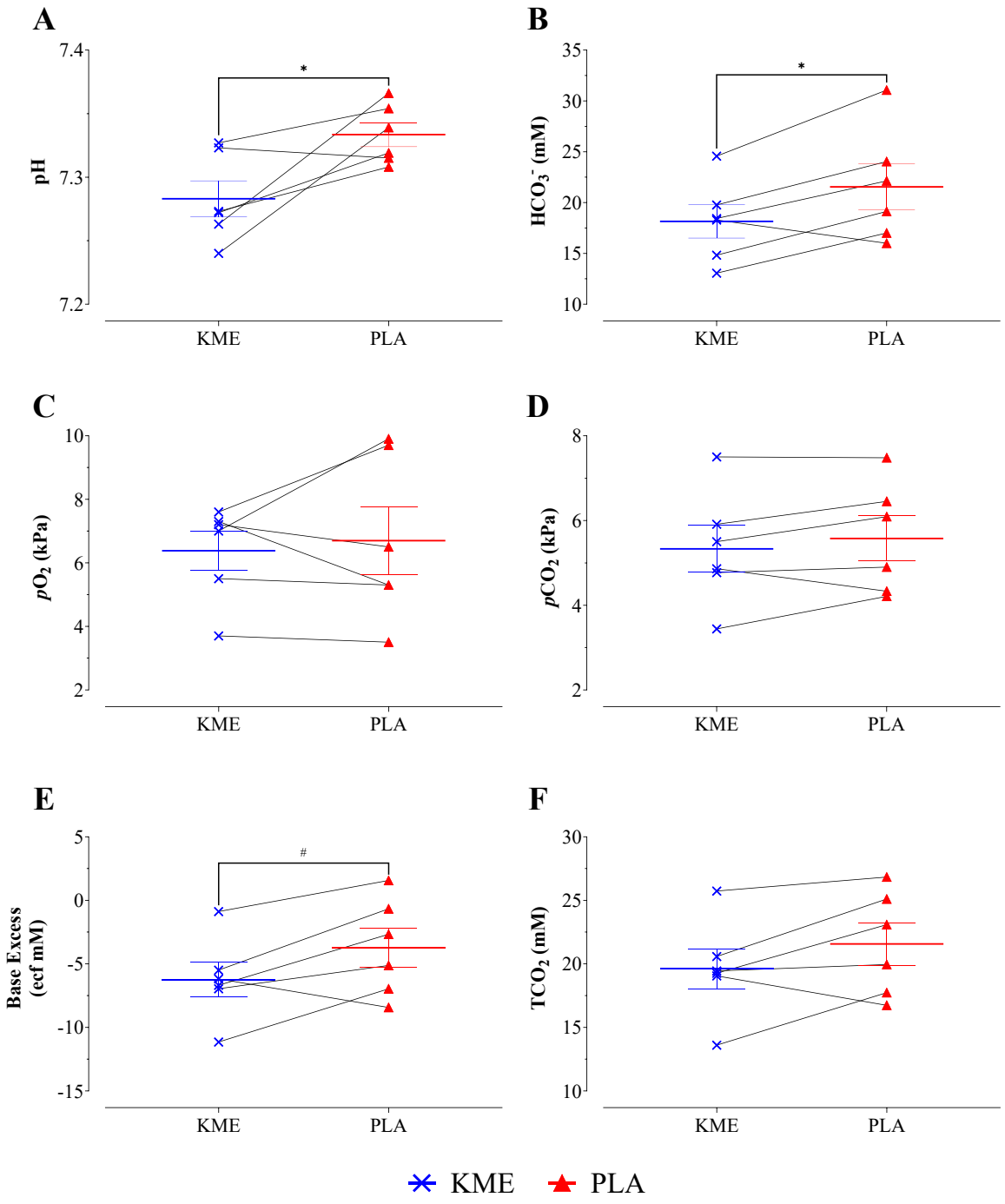


***Figure 4.20* - Venous whole blood gas measures upon completion of the TTE trial under the KME & PLA conditions.**

__A__, pH; __B__, Bicarbonate (HCO_3^-; mM); __C__, Partial Pressure of Carbon Dioxide (pCO_2; kPa); __D__, Partial Pressure of Oxygen (pO_2; kPa); __E__, Base Excess (BE; ecf mM) __F__, Total CO_2 (TCO_2; mM). TTE, time-to-exhaustion. Data presented as Mean ± SEM with individual values plotted. $^{\#}$ $p < 0.10$, * $p < 0.05$ between KME and PLA conditions. n = 6.

4.3.7 Urine Biochemistry

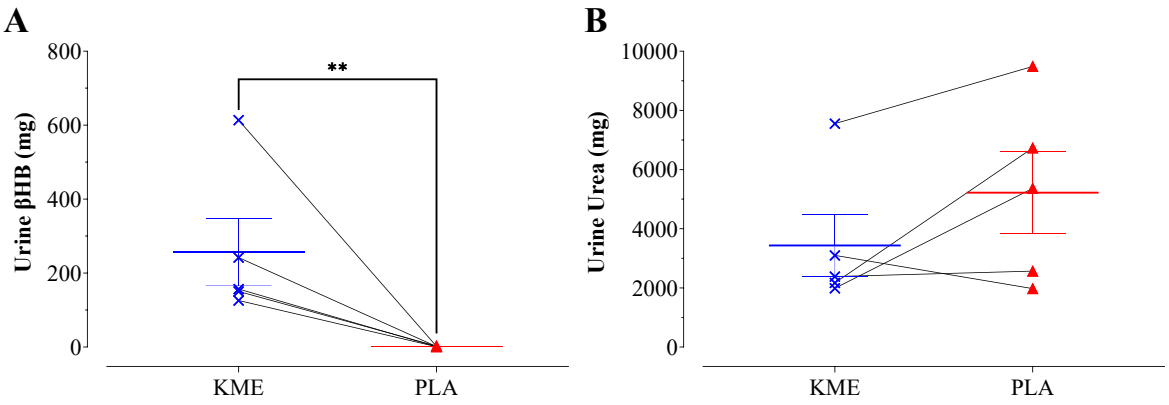


***Figure 4.21* - Urine metabolite total masses excreted from 2 hr onwards under the KME & PLA conditions.**

***A**, βHB (mg); **B**, urea (mg) total mass excreted. First KME/PLA drink consumed at 2 hr. Data presented as Mean ± SEM with individual values plotted. ** $p < 0.01$ between KME and PLA conditions. $n = 5$.*

Total urinary βHB mass from the point of ingestion of the first KME/PLA drink (2 hr) onwards was found to be greater under KME compared to PLA ($p = 0.004$; *Figure 4.21-A*). Plasma ΔβHB-AUC did not predict urine ΔβHB excretion ($p = 0.549$). There were no between-condition differences for urea mass excreted ($p = 0.158$; *Figure 4.21-B*) nor for total urine volume ($p = 0.444$).

4.3.8 Gastrointestinal Distress, Appetite, & Perceived Exertion

Fasting

No differences between condition were found for any appetite measure nor gastrointestinal distress at the commencement of each visit whilst overnight fasted (Appendix K).

4 hr Protocol

Gastrointestinal distress exhibited a time ($p = 0.008$; *Figure 4.22*) and interaction ($p = 0.049$) effect, in the absence of an effect of condition ($p = 0.103$), across 0 hr to 4 hr.

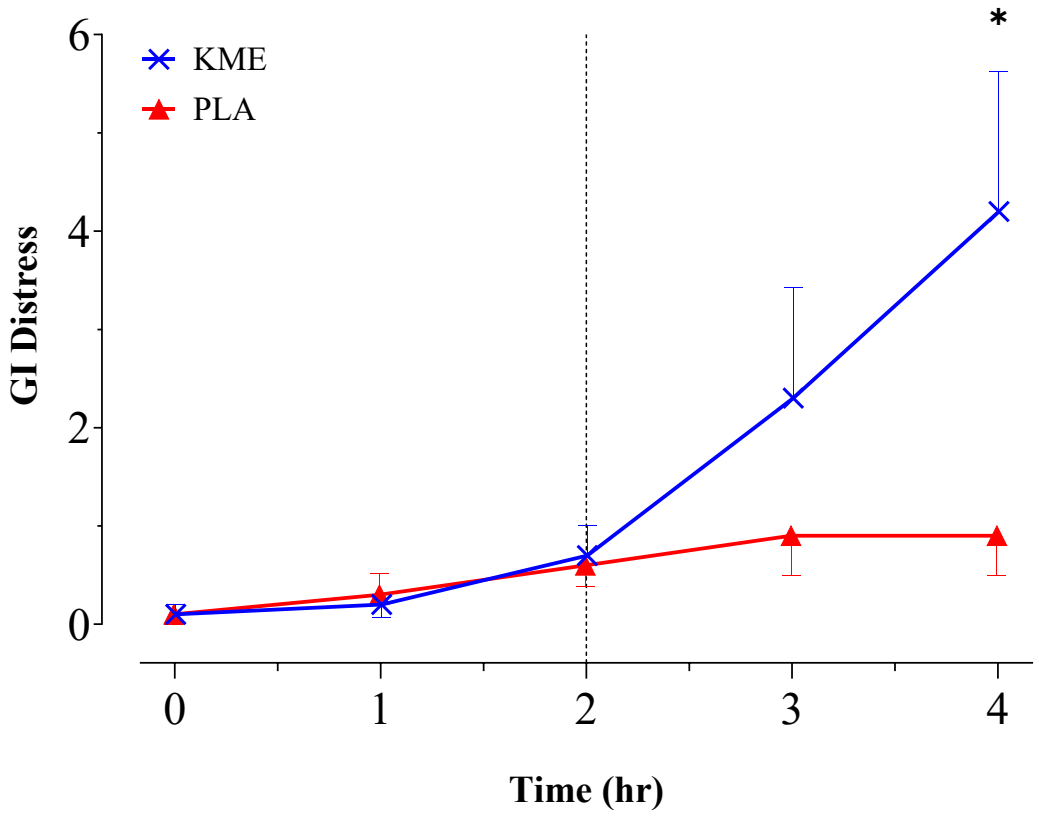


***Figure 4.22* - GI distress, during the 4 hr cycle protocol under the KME & PLA conditions.**
Gastrointestinal (GI) distress, sum of symptom scores for each of the 12 specific measures at a given timepoint. Data presented as Mean ± SEM. Dashed line, first KME/PLA drink (given immediately after 2 hr timepoint). * *p < 0.05 between KME and PLA conditions at a given timepoint. n = 10.*

No condition or interaction effects were seen for any of the appetite metrics ($p \geq 0.176$; Appendix K). *Satisfied* exhibited an effect of time ($p = 0.007$), whilst trends for effects of time were seen for *Hunger* ($p = 0.089$) and *Total Appetite* ($p = 0.053$). *Fullness* and *How Much Could Eat* did not display time effects ($p \geq 0.404$).

General, *Breathlessness Intensity*, *Leg Discomfort*, and *Anxiety of Leg Discomfort* RPE levels all presented effects of time ($p \leq 0.037$; Appendix L), whereas no time effect was seen for *Anxiety of Breathing* ($p = 0.233$). No condition or interaction effects were established for any measures ($p \geq 0.223$), except for a weak trend a condition effect being observed for *Anxiety of Breathing* indicating levels might have been greater under KME ($p = 0.082$).

TTE Completion

No effect of condition was found for any perceived exertion measure upon completion of the TTE trial (Appendix L).

4.3.9 Blinding Efficacy

Of the ten participants, four correctly identified visit condition order, reporting an average certainty in that choice of 2.0. Those who guessed incorrectly provided an average certainty rating of 3.5. Thus, condition blinding appeared to be efficacious.

4.3.10 Exploratory Analysis - Gross & Delta Efficiency and Substrate Oxidation

Below is analysis conducted using equations for substrate oxidation[257] which are generally considered to be confounded by a state of exogenous ketosis[69]. From these substrate oxidation rates, EE and therefore gross & delta efficiency were calculable. Results are presented both without any correction for ketone oxidation (*Ketone Uncorrected*) and with an assumption of 0.10 g/min βHB oxidation[71] (*Ketone Corrected*).

Efficiency

Ketone Uncorrected gross economy tended to fall (worsen) over time ($p = 0.091$; *Figure 4.23-A*), with no condition or interaction effects ($p \geq 0.211$) present. *Ketone Corrected* analyses observed gross efficiency under KME to be increased across the exogenous ketosis period (2 hr 30 min to 4 hr) by 0.01-0.02% compared to *Ketone Uncorrected*, which did not influence statistical outcomes (data not shown).

Delta efficiency outcome data were identical between the *Ketone Corrected* and *Uncorrected* analyses, with values not changing across the 4 hr period ($p = 0.117$; *Figure 4.23-B*). Condition and interaction effects ($p \leq 0.048$) were observed though, with efficiency lower (worse) under KME compared to PLA. R^2 for the regressions used to calculate delta efficiency were >0.9 in all instances.

No time, condition, or interaction effects were seen for *Ketone Uncorrected* EE ($p \geq 0.213$; *Figure 4.23-C*), whilst *Ketone Corrected* EE was 0.6559 kcal/hr (~0.09%) greater under KME from 2 hr 30 min onwards, which did not influence statistical outcomes (data not shown).

Therefore, efficiency measures, when EE is calculated from substrate oxidation rates individually, can be assumed to be equivalent with and without a systematic correction for (expected) βHB oxidation.

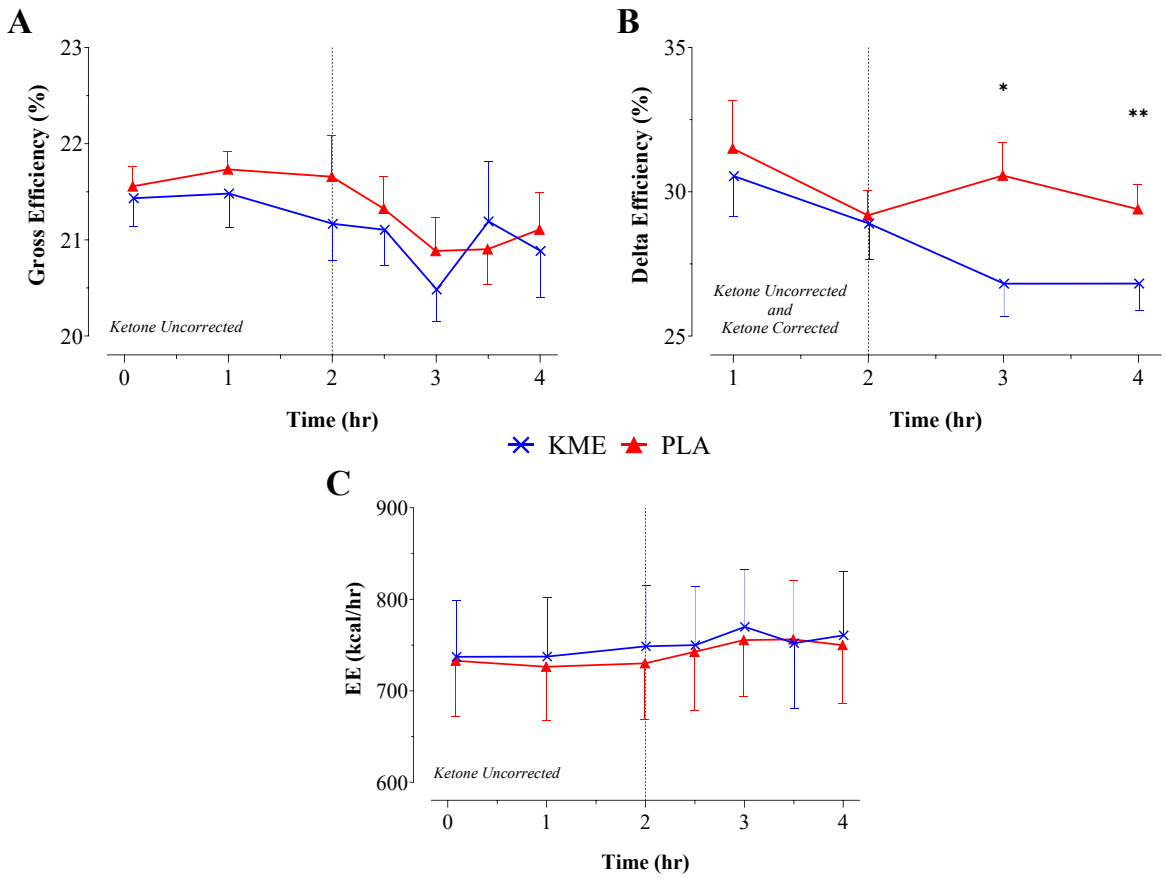


***Figure 4.23* - Exploratory analysis: cycling efficiency and energy expenditure during the 4 hr cycle protocol under the KME & PLA conditions.**

***A**, Gross Efficiency (%), Ketone Uncorrected analysis; **B**, Delta Efficiency (%), Ketone Corrected and Uncorrected analysis (identical outcome data). **C**, Total Energy Expenditure (EE; kcal/hr), Ketone Uncorrected analysis. Data presented as Mean ± SEM. Dashed line, point of first KME/PLA drink (consumed immediately after 2 hr timepoint). * $p < 0.05$, ** $p < 0.01$ between KME and PLA conditions at a given timepoint. $n = 8$.*

Substrate Oxidation

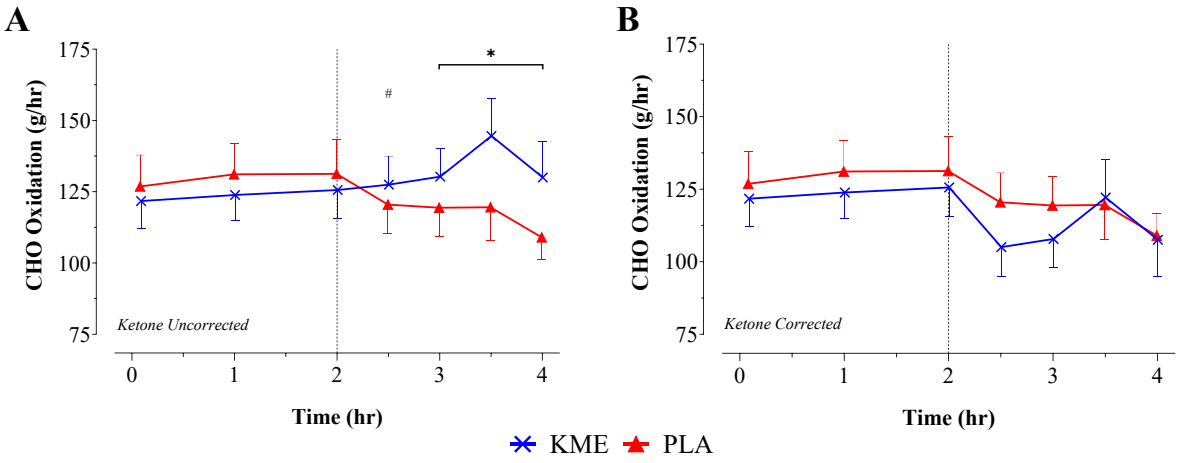


***Figure 4.24* - Exploratory analysis: carbohydrate oxidation rates during the 4 hr cycle protocol under KME & PLA conditions.**

*A, Ketone Uncorrected analysis; **B**, Ketone Corrected analysis. CHO, carbohydrate. Oxidation rates as grams per hour (g/hr). Data presented as Mean ± SEM. Dashed line, point of first KME/PLA drink (consumed immediately after 2 hr timepoint). # $p < 0.10$, * $p < 0.05$ between KME and PLA conditions at a given timepoint. n = 8.*

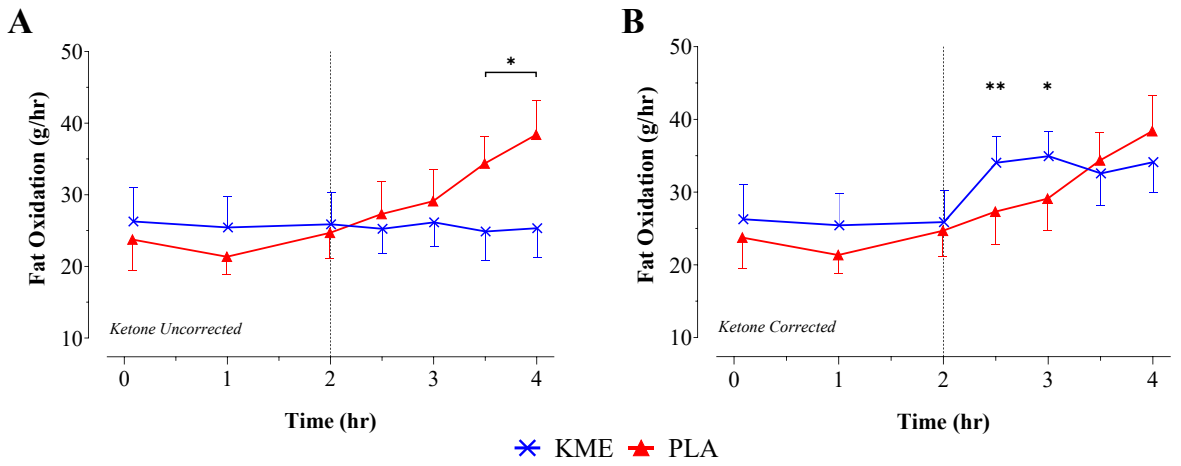


***Figure 4.25* - Exploratory analysis: fat oxidation rates during the 4 hr cycle protocol under the KME & PLA conditions.**

*A, Ketone Uncorrected analysis; **B**, Ketone Corrected analysis (exploratory post-hoc analyses as weak trend for an interaction effect seen). Oxidation rates as grams per hour (g/hr). Data presented as Mean ± SEM. Dashed line, point of first KME/PLA drink (consumed immediately after 2 hr timepoint). * $p < 0.05$, ** $p < 0.01$ between KME and PLA conditions at a given timepoint. n = 8.*

Ketone Uncorrected analysis exhibited no effect of time for carbohydrate oxidation ($p = 0.613$; *Figure 4.24-A*), but an interaction effect was found ($p < 0.001$) alongside a trend for a condition effect ($p = 0.082$). *Ketone Corrected* analysis revealed trends for effects of time ($p = 0.067$; *Figure 4.24-B*) and condition ($p = 0.092$; weak trend), but no interaction effect was found ($p = 0.332$).

Ketone Uncorrected analysis showed no effects of time or condition ($p \geq 0.103$; *Figure 4.25-A*) for fat oxidation, however an interaction effect was present ($p < 0.001$). Under *Ketone Corrected* analysis, a time effect ($p = 0.006$; *Figure 4.25-B*) was present, but no condition effect was found ($p = 0.230$). As this was exploratory analysis, a weak trend for an interaction effect ($p = 0.081$) was deemed sufficient to permit exploratory post-hoc tests.

By contrasting *Ketone Corrected* to *Uncorrected* results, it can be concluded that when (expected) βHB oxidation rates are systematically used to correct indirect calorimetry data, substrate oxidation rates substantially differ compared to when no such corrections are attempted. Thus, it appears that the accuracy of uncorrected rates cannot be confidently assumed under exogenous ketosis.

Energy Expenditure

When EE was calculated using *Ketone Corrected* analysis, under exogenous ketosis (KME condition, 2 hr 30 min to 4 hr) values were 3.56% greater (min: 2.55%, max: 5.49%; Bland Altman analysis, bias: +38.42 ± 9.01 kcal/hr, 95% Limits of Agreement: -11.51 to +88.38 kcal/hr) when calculated from individual substrate oxidation rates, as was the method employed in this thesis, compared to the singular equation proposed by Jeukendrup and Wallis[257], that has been used in a number of exogenous ketosis studies[70,71,94,153,230].

Thus, comparisons between EE, and therefore efficiency, datasets generated via differing calculation methods should be approached with caution if βHB oxidation correction is attempted.

4.4 Discussion

This study explored whether KME ingestion might improve the resilience of moderate intensity cycling economy, and how subsequent high intensity work capacity may be influenced, in a well-trained endurance cyclist population. Gross economy and TTE performance did not differ between exogenous ketosis and placebo conditions. Delta economy was, however, worsened under KME compared to PLA despite findings of higher RER values, indicative of proportionally lesser reliance on oxygen-inefficient FA oxidation, whilst exogenous carbohydrate oxidation rates were supressed.

4.4.1 Exercise Economy

The primary outcome of this work was cycling economy, assessed in both gross and delta forms[250,252], across the 4 hr moderate intensity protocol. It was hypothesised that a low-carbohydrate state would be achieved after ~2 hr, potentially permitting exogenous ketone oxidation to more tangibly contribute to EE through diminished competitive from glucose[223]. In turn, this would act to arrest an expected progressive worsening in economy, through ketolysis supplanting a portion of an otherwise elevated reliance on β-oxidation (P/O: Glucose, 2.58; βHB, 2.50; FA, ~2.33)[91,98].

A shift towards greater lipid utilisation was indeed observed across the 4 hr protocol, with fat oxidation rates during PLA nearly 2-fold greater at 4 hr compared to during the first hour. Whilst not achieving statistical significance in the whole cohort (though doing so in the n = 5 participants who were provided ^{13}C tracer), carbohydrate utilisation commensurately declined from ~125-130 g/hr across 0-2 hr, to 109 g/hr by 4 hr. Despite this substrate shift, delta economy did not change over time, whilst gross economy only exhibited a tendency to worsen across the 4 hr. This sits in contrast to prior work where impaired economy/efficiency was present after ≥2 hr of cycling[248,249,462,481], though findings are not universal[252,482].

Within this lowered carbohydrate oxidation setting from ~2 hr onwards, delta economy was worsened by ~10% under exogenous ketosis, which was introduced at the 2 hr timepoint, compared to PLA. This sits in contrast to work by Dearlove et al.[71] who observed a ~7% improvement (relative change) in cycling delta efficiency after KME ingestion, compared to placebo, in six fasted participants. As economy and efficiency measures do not necessarily always correlate[99,250], exploratory analyses were

undertaken in this chapter to determine gross and delta efficiency using the βHB oxidation rates reported by Dearlove et al.[71], who employed a ^{13}C-KME tracer. Delta efficiency, in this exploratory work, was found to be worsened by exogenous ketosis, whilst gross efficiency was not different between conditions, mirroring findings seen for economy measures.

Gross economy, and potentially also gross efficiency, being unaffected by KME ingestion in my study aligns with Bone et al.[94] who explored cycling efficiency measures in large cohort (n = 28). They found no effect of the KME, at either a high (~3 mM plasma βHB) or low (~2.3 mM) dose, on cycling gross economy at 75%, 100%, or 125% VT1 in a postprandial state without in-exercise carbohydrate provision, compared to placebo, whilst delta and gross efficiency outcomes were also determined to be unaffected. However, as it is not detailed how KB oxidation rates were accounted for when determining EE in these studies by Bone et al.[94] and Dearlove et al.[71], it is unclear how directly comparable their efficiency findings are to each other's, and to my exploratory analyses.

Findings across the wider literature tentatively indicate that benefits of exogenous ketosis for exercise economy might only exist between ~1.5-2 mM, recapitulating the ergogenic 'optimal range' concept from *Chapter 3*, and potentially explaining my observations at 1.98-2.82 mM βHB here. In support of this hypothesis, Brady & Egan[232] observed improved running economy compared to placebo at 1.8-2.1 mM plasma βHB, but not at 2.5-3.0 mM βHB, across identical testing protocols[233], whilst in aforementioned work by Dearlove et al.[71] cycling delta efficiency was improved at 1.73-1.87 mM βHB, but not at 4.1-4.33 mM. Furthermore, no effect of postprandial exogenous ketosis was found on gross economy during intermittent cycling at >2 mM βHB[157], race walking at ~3 mM[457], nor 65% $\dot{V}O_{2max}$ running at 1.0-1.3 mM[154]. Fasted cycling economy was similarly unaffected by ketosis at >2 mM βHB in all sub-studies presented by Cox et al.[107], whilst 4.0 mM βHB did not influence post-exercise cardiac or quadriceps high-energy phosphate metabolism in 24 hr fasted participants[483]. However, only four studies have been designed around economy, or efficiency, as a primary outcome measure[71,94,232,233], and broader data is too sparse and heterogenous regarding exercise modality, intensity, and population studied to draw any firm conclusions.

4.4.2 Substrate Oxidation During the 4 hr Protocol

The rationale for exogenous ketosis enhancing cycling economy centres around βHB oxidation, with its superior P/O[69,91], supplanting β-oxidation. Whilst exogenous ketosis confounds calculation of absolute substrate oxidation rates from indirect calorimetry[69,257], RER still provides insight into their relative contributions to EE. Observations of greater in-exercise RER after KME ingestion in the present work sit in contrast to prior findings of lower values[107,232,233] or no effect[125,154,157,230]. However, no study has explored exogenous ketosis (with βHB remaining >1 mM[146,157]) in exercise >2 hr in duration where RER might be expected to decline[240,482]. Higher RER under KME compared to PLA in my work appears to be consequent of greater $\dot{V}CO_2$, with $\dot{V}O_2$ not differing. This was not consequent of ketoacidosis generating additional CO_2 via HCO_3 buffering, as corrections were made for this, therefore between-condition differences appear to reflect dissimilar substrate metabolism.

During PLA, RER fell from 2-4 hr, with elevated fat oxidation reflected in plasma NEFA levels markedly rising to increase provision to exercising SM, and IMTAG lipolysis rates likely also increasing[198,243,246]. Reciprocally, total carbohydrate oxidation fell, potentially to spare diminishing SM and hepatic glycogen stores[243] as evidenced by a progressively lowered contribution of endogenous carbohydrate to total carbohydrate oxidation. In contrast, RER under exogenous ketosis didn't decline in this period and was greater or tended to be greater, at all timepoints (range, 0.901-0.916; average, 0.905) compared to PLA. As FAs are the only substrate with a respiratory quotient (RQ) <<0.9, the proportional contribution of lipid oxidation to overall EE must have therefore been lower under KME compared to PLA. It is possible that KB oxidation underpinned this elevated RER, which was similar to the RQ of βHB, and below that of AcAc, under exogenous ketosis (RQ: βHB, 0.89; AcAc, 1.00)[69]. However, oxidation of βHB, which would be expected to be circulating at ~twice the concentration as AcAc[125], likely only contributed ~4%[71] to EE, and certainly not >10%[70], based on stable isotope work in trained athletes cycling in a fasted, i.e. similarly low-carbohydrate, state at similar intensities. As glucose's RQ is 1.00, it is therefore more probable that it was proportionally increased carbohydrate oxidation driving these elevations in RER under KME.

If EE was the same between conditions, a reduced relative contribution of β-oxidation under KME should have improved cycling economy compared to PLA[91]. As this was not the case, it appears that,

though there was a greater proportional contribution of more oxygen efficient substrates (glucose and/or KBs) to oxidative phosphorylation under exogenous ketosis, ATP production and/or utilisation was less efficient. Therefore, greater absolute amounts of substrate, likely primarily carbohydrate, would be required to offset these inefficiencies, increasing oxygen demands for the same functional power output.

As delta economy was worsened by ketosis, whilst gross economy was unaffected, these inefficiencies were likely localised within oxygen-demanding working musculoskeletal systems[68], namely SM and cardiorespiratory (discussed in 4.4.4) physiologies. Concerning working SM, at the moderate exercise intensities examined here leg $\dot{V}O_2$ likely accounted for ~55% of whole body oxygen consumption[484] with power produced primarily by type I ('slow twitch') fibres[197,485]. Therefore, it is within these highly oxidative fibres where contractile efficiency appears to have been worsened by exogenous ketosis. These inefficiencies likely also caused premature fatigue of these fibres, resulting in progressive recruitment of glycolytically dominant type II ('fast twitch') fibres to compensate. As type II fibres are oxidatively less efficient[254,486–489], this shift is strongly associated with the development of a $\dot{V}O_2$ slow component, i.e. increased oxygen utilisation for the same output, reflected in impaired exercise efficiency/economy[252,253,486,487].

As exogenous carbohydrate oxidation was lowered under KME, it appears that the posited increased total carbohydrate oxidation may have been disproportionately reliant on endogenous sources. With circulating glucose availability lowered during ketosis, likely due to reduced hepatic output[37], SM would be heavily dependent on intra-muscular glycogen stores for glucose provision. Though exogenous ketosis was determined to supress SM glycogenolysis by Cox et al.[107], this was established during fasted exercise, exogenous carbohydrate provision was not matched between conditions, and the histological methodologies employed have been debated[137,146,179]. Conversely, Poffé et al.[146] observed a trend ($p = 0.08$) for glycogen depletion to be greater under exogenous ketosis compared to a non-caloric placebo across ~3 hr of intermittent (moderate and heavy intensity) postprandial cycling with 60 g/hr of carbohydrate. Alongside this, plasma [βHB] positively ($r = 0.58$), and plasma [FA] negatively ($r = -0.45$), correlated with the rate of glycogen depletion, reflecting an inverse relationship between glycogenolysis and circulating FA availability that has been observed in non-ketotic settings[202,204,490,491].

Based on these observations, I propose a mechanism, detailed in *Figure 4.26*, wherein SM glycogenolysis remains elevated under exogenous ketosis when it would normally be progressively supressed during exercise >2 hr by declining glycogen levels, resultant of the unique physiological setting of prolonged lowered glucose and FA, alongside elevated ketone, circulating availability.

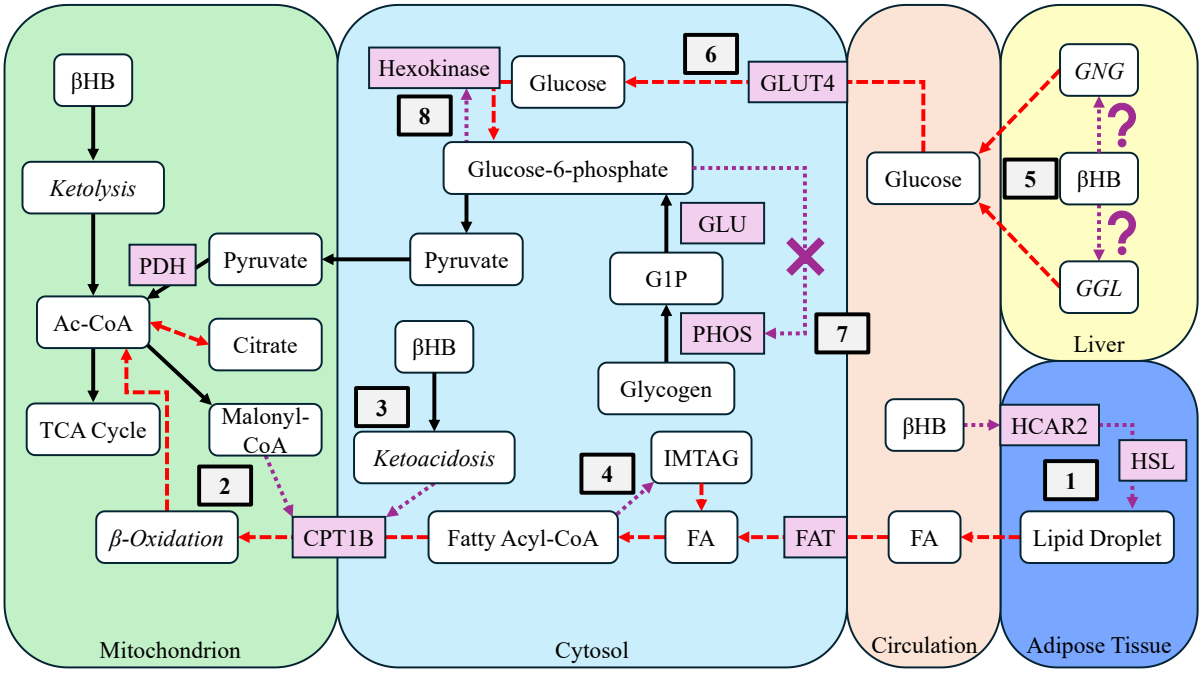


***Figure 4.26* - Proposed mechanism by which exogenous ketosis may increase reliance on glycogenolysis, and reduce β-oxidation, in skeletal muscle at moderate exercise intensities.**

Red line with long dashes, downregulated pathway.

Purple line with short dashes, inhibition of an enzyme or pathway.

Purple line with short dashes and a cross, disinhibition of an enzyme or pathway.

Purple line with short dashes and a question mark, potential inhibition of a pathway.

Compared to a non-ketotic state, exogenous ketosis might directly and indirectly act to reduce β-oxidation, and potentially plasma glucose oxidation, within exercising SM, with glycogenolysis consequently increasing to meet the high energetic demands of exercise[492]*:*

1. Circulating FA levels are lowered by βHB inhibiting adipose hydroxycarboxylic acid receptors (HCAR2)[105,493]*, supressing hormone-sensitive lipase (HSL) driven droplet lipolysis*[195,494] *(even when stimulated by elevated catecholamines*[495]*). As lipid oxidation in skeletal muscle (SM) during exercise is positively associated with FA availability in circulation*[202,490]*, even alongside elevated insulin*[460] *(which is equivocally increased during exogenous ketosis*[37,107]*), low availability of plasma FAs for uptake by FA transporters (FAT; e.g. FABP$_{PM}$/CD36/FATP*[145]*) acts to supress β-oxidation that would otherwise be increasingly relied on as SM & hepatic glycogen stores are progressively depleted*[134,197,240,258]*.*

2. Accumulation of mitochondrial acetyl-CoA (Ac-CoA)[73]*, consequent of ketone and glucose oxidation, increases malonyl-CoA levels which allosterically inhibits carnitine palmitoyltransferase 1B (CPT1B)*[496–498]*, bottlenecking transport of long-chain fatty acyl-CoA into the mitochondria and reducing availability of acyl carnitines for β-oxidation. Less Ac-CoA and citrate is therefore produced from β-oxidation, disinhibiting pyruvate dehydrogenase (PDH) and phosphofructokinase (PFK)*[132,204]*, thereby*

increasing glycolytic flux capacity. Exogenous ketosis has been observed to decrease the acetyl-carnitine/free carnitine ratio in exercising SM, potentially reflecting this suppression of CPT1B[107,499]*, whilst ketosis also accelerates AMPK deactivation immediately post-exercise without de-inhibiting glycogen synthase*[171]*, which would further elevate malonyl-CoA levels*[500] *if this also occurs in-exercise. Additionally, βHB is known to bind to free carnitine, elevating intra-muscular C_4-OH-carnitine ('keto-carnitine')*[107]*, though it is unclear if this impacts lipid metabolism*[247]*.*

3. Ketoacidosis independently impairs lipid bioenergetics by attenuating respiration with L-carnitine and palmitoyl-CoA, and enhancing the sensitivity of CPT1B to inhibition by malonyl-CoA[501]*.*

4. Fatty Acyl-CoA accumulates upstream of reduced CPT1B flux. As it is an allosteric inhibitor of HSL[500,502] *this supresses intra-muscular triacylglycerol (IMTAG) lipolysis, a process that would normally be upregulated by low glycogen and lowered plasma FA availability*[204]*.*

5. Ketosis reduces hepatic glucose output[150]*, potentially via inhibition of gluconeogenesis (GNG) and/or glycogenolysis (GGL). Though the underlying mechanisms are unclear, diminished circulating glycerol*[178] *levels may act to supress gluconeogenesis.*

6. As glucose uptake into SM is primarily via GLUT4, a passive transporter[135]*, lowered circulating glucose concentration diminishes the extra-to-intracellular diffusion gradient, slowing influx and reducing rates of conversion to glucose-6-phosphate (G6P).*

7. During glycogenolysis, glucose-1-phosphate (G1P) is cleaved from glycogen by glycogen phosphorylase (PHOS). G1P is then converted to G6P by phosphoglucomutase (GLU), with G6P entering the glycolytic pathway[134,305,503]*. Lowered appearance of G6P from glucose reduces allosteric inhibition of PHOS, whilst also deactivating glycogen synthase*[134,277,504,505]*. Both ADP:ATP and AMP:ATP are key indicators of cellular energy status, which are increased by the high ATP demands of muscle fibre contraction, especially with low circulating substrate availability*[506] *under ketosis. PHOS activity is therefore indirectly elevated by increased ADP:ATP stimulating phosphorylase kinase*[507]*, whilst being directly allosterically activated by greater cytosolic AMP:ATP*[508]*. Low intracellular glucose during ketosis additionally activates PHOS by reducing sAC-cAMP-Epac1 signalling*[509]*, independent of hormones such as glucagon & adrenaline which are equivocally influenced by KME ingestion (see Chapter 3).*

8. Accumulation of G6P from disinhibited glycogenolysis potentially inhibits hexokinase activity, which can occur at low cytosolic glucose concentrations[134]*, supressing glycolysis of plasma glucose.*

Accelerated depletion of glycogen stores under exogenous ketosis might then explain how type I SM fibres could have been prematurely fatigued, with type I glycogen depletion previously observed to increase $\dot{V}O_2$ by 7-9% through elevated type II fibre recruitment[510]. This shift is posited to be resultant of depletion of intramyofibrillar subcellular glycogen pools[143,511–513] in particular, with consequent reduced sarcoplasmic reticulum (SR) Ca^{2+} release, slowed fibre relaxation rates, lowered membrane excitability, and reduced Na^+/K^+-ATPase function[397,488,512] contributing to impaired excitation-contraction coupling, thus diminished power generation capacity. Whilst these mechanisms can be driven by a low energy state[399], impairment of muscle function by low glycogen also occurs independent of ATP availability, with structural changes to glycogen granules physically dissociating glycogenolytic

complexes from the SR[397], indicating that reduced contractility under low glycogen might be obligate. Therefore even if oxidative substrate provision were to increase, for example, consequent of severely depleted glycogen being unable to sufficiently provision glycolysis, reducing malonyl-CoA accumulation and thus disinhibiting IMTAG lipolysis[246,340] (*Figure 4.26, Points 2 & 4*), loss of function would not be rescued.

As type II fibres are highly glycolytic[197], their increased recruitment, alongside proposed increased glycogenolysis, might have been expected to elevate circulating lactate. This was not seen here, potentially explained by saturation of MCTs by KBs reducing lactate efflux rates[155,156]. Additionally, increased type II fibre recruitment resultant of type I fibre glycogen depletion has previously been observed to occur in the absence of changes to plasma lactate[510].

Reduced exogenous carbohydrate oxidation under exogenous ketosis, as was found in work presented here, would further increase reliance on, and thus depletion of, endogenous carbohydrate stores. The only other study to have employed a ^{13}C-carbohydrate tracer during exercise under exogenous ketosis is that of Howard et al.[150], who observed no effect of the KME on exogenous nor plasma glucose oxidation. Though exercise intensities were roughly similar between their work and the protocol utilised in this chapter, Howard et al.[150] explored weighted treadmill exercise, carbohydrate was provisioned at 30 g/hr, and participants were all male and less well-trained ($\dot{V}O_{2peak}$ ~50 mL/min/kg), which may explain the discordant findings. Lowered exogenous carbohydrate oxidation in my work is unlikely to have been consequent of reduced glycolytic flux[31,107,128] (*Figure 1.5*) as this would have supressed all carbohydrate oxidation and therefore likely lowered, not raised, RER. It may reflect slowed gastric emptying/transit, something that is known to occur after direct βHB ingestion[514] and is posited to occur with the KME itself[23,515], but has not yet been observed[150,158,416]. Alternatively, circulating insulin levels may have been elevated under ketosis[107] here, increasing hepatic glucose uptake and storage, though insulin levels are equivocally modified by the KME[37] during exercise and were not different between conditions in *Chapter 3*. The observed average 4.1 g/hr reduction in exogenous carbohydrate oxidation under KME compared to PLA does, however, only represent 3.3% of the total carbohydrate oxidation in PLA, thus its impact on overall substrate utilisation is unlikely to have been significant.

Future work might endeavour to directly quantify glycogenolysis during prolonged moderate intensity exercise to investigate the effects of exogenous ketosis proposed here. As the assessment of SM glycogen by biopsy histology is debated[107,137,146,150], dual-tracer approaches, with labelled carbohydrate and KME, might instead be utilised[70,71,516]. Furthermore, near-infrared spectroscopy (NIRS)[237,248,517] could be employed to evaluate SM oxygen utilisation, which might better characterise changes to economy seen here.

Circulating glucose suppression may have additionally contributed to worsened economy by accelerating central fatigue, wherein impaired motor unit recruitment patterns could compromise muscle contractile function, necessitating greater recruitment of type II muscle fibres[518,519] to compensate. This could be explored through use of surface electromyography (sEMG)[520–522].

As economy of the working musculoskeletal system[250,252] was worsened under exogenous ketosis, but whole body economy was unaffected, this indicates $\dot{V}O_2$ was commensurately reduced in basal systems (i.e. those whose metabolic rate did not change proportional to exercise intensity) to offset this worsened delta economy. Energetic turnover might have been made more oxygen efficient through ketones being oxidised in place of lipids in highly ketolytic organs like the kidneys[38]. Oxygen demanding processes could have also been supressed. For example, gluconeogenesis from alanine[69] is elevated during exercise[362] but lowered by exogenous ketosis[136,150]. Additionally, ketogenesis, which is directly oxygen consuming, appeared to be elevated in PLA with plasma βHB rising 3-fold from 2-4 hr, but would have been supressed under KME[53,54]. Protein catabolism[523,524] may have also been lower under KME, with ketosis posited to be anti-catabolic[171,187,188] and a trend for lowered plasma urea[1] seen here. Increased oxidation of amino acids under PLA could have also contributed to RER being lower. However, only 13.57 g of protein[525] was oxidised in PLA, equating to 55.64 kcal[526], <3.5% of EE.

4.4.3 Circulating Acid-Base Levels During the 4 hr Protocol

SM inefficiencies under KME, potentially consequent of altered substrate availability, may have been augmented by ketoacidosis which itself can adversely impact contractile efficiency. Circulating acidosis after KME ingestion is a robustly observed phenomenon[23,262] and is especially prevalent during exercise

where buffering systems in place to maintain acid-base homeostasis[264] are already taxed[71,152,153,157,237]. In my work, the observed acidosis appeared to be directly consequent of ketosis, with plasma [βHB] predicting 55.1% of the reduction in base excess under KME compared to PLA. Though pH changes in the bloodstream do not necessarily influence the intra-/peri-muscular milieu, elevated [H^+] is generally considered to carry ergolytic potential[264]. If ketoacidosis impaired type I fibre efficiency, this might have led to increased oxygen use in these fibres, as well as a compensatory increase in type II fibre recruitment, amplifying the same dynamic that was potentially also being driven by glycogen depletion.

Whilst the influence of elevated H^+ on exercise efficiency/economy at moderate intensities is poorly understood[264,267], acidosis has been shown to reduce the oxidative efficiency of SM mitochondrial ATP production[527], potentially through increased uncoupling via UCP3[278], though other work shows no impact of acidosis on mitochondrial function[260,501]. At a muscle fibre level, acidosis disrupts contractile function[270], whilst additionally reducing the free energy change of ATP hydrolysis[528] and potentially depleting high energy phosphate stores, something that could be explored in future work utilising ^{31}P-MRS imaging[529–531]. These detrimental impacts of acidosis may explain why Brady & Egan[232]/Brady et al.[233] saw improved running economy at 1.8-2.1 mM plasma βHB[232], but not at ~2.5-3 mM[233], with increased ketoacidotic load nullifying any ketolysis-driven increase in oxidative efficiency in the latter study. Direct comparison of these findings to my work should be approached with caution though, as acidosis may act more detrimentally in cycling than in running[402].

4.4.4 Cardiorespiratory Measures During the 4 hr Protocol

Alongside potentially impairing muscular contractile function, acidosis is known to increase ventilatory drive, which itself carries an oxygen cost. Elevated ventilatory rates under exogenous ketosis, seen in this chapter and in previous work[125,153,157], are likely driven by ketoacidosis[230]. In my work V_E was elevated by 6.36 L/min under KME compared to PLA, consequent of increased V_T, contrasting with *Chapter 3* where changes to V_E appeared to be driven instead by elevated BF. This is consistent with other literature, with increased V_E found to be consequent of elevated BF at higher exercise intensities[125,157], but driven instead by greater V_T during exercise in proximity to the moderate-heavy intensity domain boundary[94,125,230]. This might be explained by V_T's limited scope to be increased at

high intensities[532], though the influence of exercise modality on breathing patterns[533], for example when comparing this work and *Chapter 3*, cannot be discounted.

Cardiorespiratory system oxygen utilisation increases in line with exercise intensity[534] and therefore alterations to this relationship would potentially influence delta economy. Increased V_E under KME in work presented here, for example, would likely elevate the oxygen cost of breathing through greater diaphragm and external intercostal workloads[283,284,535]. Raised ventilatory oxygen costs, however, only explain 4.69 ± 2.83% and 10.15 ± 3.60% of the increase in delta economy from PLA to KME seen at 3 hr and 4 hr, respectively, when calculated using comparable $\Delta\dot{V}O_2/\Delta W$ values from Aaron et al.[282]. Greater ventilatory workload under ketosis has previously been associated with elevated HR[125,146,229,230,283,284,287], though the KME has also been observed to not affect HR[151,152,237], as was the case in my work presented in this chapter, or even cause a slight decrease[232]. Therefore, increased cardiorespiratory system oxygen utilisation appears to represent a tangible, but relatively minor, determinant of delta economy having worsened during exogenous ketosis, indicating that impairments to efficiency occurred primarily at a working SM level.

Increased type II fibre recruitment consequent of progressive type I fatigue, especially under KME, likely led to the power associated with the moderate-heavy intensity domain threshold falling across the 4 hr protocol in my work, with VT1 previously found to have decreased by 3% after 180 min of 90% VT1 cycling with the same carbohydrate provision as here[536]. Therefore, the highest power within the step protocol used to determine delta economy ('+15W': 97.6 ± 0.7% LT1; *Figure 4.4*) may have fallen into the heavy domain as participants fatigued, with the subsequent appearance of a $\dot{V}O_2$ slow component[252,253] potentially contravening the assumption of $\dot{V}O_2$-power linearity[250]. However, a steady state appeared to be present for all steps, with no $\dot{V}CO_2$-$\dot{V}O_2$ or V_E-$\dot{V}O_2$ breakpoints observed[358,537].

4.4.5 Time-to-Exhaustion Trial

The secondary outcome of this study was a TTE trial at VT2 power, conducted after the 4 hr protocol, with no influence of exogenous ketosis found. This aligns with previous findings of exogenous ketosis not impacting performances for running ramp test TTEs following 40 min at 60-80% $\dot{V}O_{2peak}$[232,233], a 10

km running TT after 1 hr at 65% $\dot{V}O_{2max}$[154], a 3 kJ/kg·BW cycling TT after 30 min at VT1 (~71% $\dot{V}O_{2peak}$)[125], nor a 10 km cycling TT after a 30 min moderate intensity preload[239]. Furthermore, several studies have found KME ingestion to be detrimental to pre-loaded higher intensity exercise, with running TTE at 85% $\dot{V}O_{2peak}$ after 90 min at ~54% $\dot{V}O_{2peak}$ reduced by 10%[150] and cycling ramp test TTE impaired after both 30 min at VT1[230] and 5 min at each of 75%/100%/125% VT1[94]. In fact, only one comparable study, that of Cox et al.[107], has seen a performance benefit of exogenous ketosis.

Whilst TTE performance was not different between conditions in work presented here, circulating metabolite and cardiorespiratory measures were affected by exogenous ketosis. End-TTE (i.e. at the point of exhaustion) plasma lactate was lowered by 1.1 mM under KME compared to PLA, potentially consequent of ketolysis bottlenecking glycolytic flux[107], though ketoacidosis might have reduced lactate production[276,538,539] and/or diminished rates of H^+/lactate cotransport out of SM[540]. Additionally, lower glycogen in exercising quadriceps muscles is associated with reduced lactate release[541], supporting the hypothesis of glycogen depletion having been accelerated under KME compared to PLA conditions during the prior 2 hr of exercise (*Figure 4.26*). End-TTE NEFA levels were similarly supressed by ketosis, however this is unlikely to have impacted performance as the workload was heavily carbohydrate dependant (RER ≥0.97)[96], whilst $\dot{V}CO_2$ being lower under KME does not appear to indicate a shift in substrate oxidation as RER was not different between conditions.

Acidosis buffering capacity, which was supressed by ketosis, is unlikely to have been a limiting factor to high intensity work capacity[264,275] here, with end-TTE plasma lactate in PLA at ~6 mM contrasting to the >9 mM seen after a fresh TT of comparable duration[542]. This indicates that anaerobic work capacity, thus scope to produce H^+ to the point where it might trigger exercise cessation, had been diminished across the prior 4 hr.

With central fatigue[87,543] a potential driver of exhaustion in this pre-fatigued setting, HR was found to be nearly 6 bpm lower across the TTE trial under KME than in PLA. Though ketosis equivocally exerts an anti-diuretic effect[146,157,287] (as observed in *Chapter 5*), supressed HR here doesn't appear to be consequent of greater blood volume as total urine volume and haematocrit did not differ between conditions. Though the KME might supress sympathetic tone at rest[190], during exercise it appears to be elevated or unaffected[287,351,544] by ketosis, so was unlikely the cause of lower HR. Alternatively, cardiac

tissue carries a high ketolytic capacity[38,126]. With lowered circulating lactate and NEFA availability during the TTE trial under KME, the heart may have been sufficiently reliant on ketolysis to improve hydraulic efficiency[59,90,91], therefore stroke volume. As cardiac output, if $\dot{V}O_2$ is used as a proxy[545], did not appear to differ between conditions, HR would be reciprocally reduced.

4.4.6 Circulating βHB

Provisioning the KME at 1000 mg/kg·BW, the largest dose given across any acute interventional study, elevated plasma βHB to 1.98-2.82 mM. Despite this also being the first study to induce exogenous ketosis during, rather than prior to, exercise, the degree of ketosis achieved aligned with prior work[146,151,157,233].

A 3.9-fold difference between the smallest and largest βHB-AUCs under KME was observed in present work, with no participant characteristics predicting this variance. Additionally, 82-84% of βHB disappearance was unexplained, with urine not a major disposal site (0.26 ± 0.05 g excreted). As the determinants of KB kinetics profiles after postprandial KME ingestion remain poorly understood[23,110], further investigation is warranted to better inform dosing quantity and timing recommendations.

4.4.7 Gastrointestinal Distress

Gastrointestinal distress was elevated under KME compared to PLA, an effect present in previous work[125,146,151,152,229], though this elevation was only seen at 4 hr here and it is unclear if it impacted economy. If the KME ingestion does slow gastric emptying, as hormone data in *Chapter 3* might indicate, consequent fluid retention in the small bowel might explain increased upper abdominal symptoms[414,415]. The impact of these disturbances could be explored further by assessing circulating markers of intestinal dysfunction such as claudin-3, intestinal FA binding protein (I-FABP), and lipopolysaccharide binding protein (LBP)[150,546–550].

4.4.8 Strengths & Limitations

The study presented in this chapter followed current methodological recommendations for exercise nutritional research[386,445] wherever possible. Participants were well-trained competitive cyclists, pre-visit training and nutrition was standardised, blinding was success, sexes were equally represented, and pre-exercise nutrition was optimal[3,5], whilst delta economy was calculated across six workloads as is best practice[99,250]. Additionally, the protocol was the longest of any study exploring acute exercise and exogenous ketosis[31,32], with findings therefore translatable to the bike-leg of Ironman triathlon racing which is completed by professionals in ~4-5 hr[356,551,552].

This work does, however, carry limitations. Use of a TT instead of a TTE would have provided greater reliability for the high intensity test, as the expected coefficient of variation (CV) for a TTE test of this nature is 17.3-24.5%[386,553,554] whereas for a glycogen-depleted TT it is ~3.8%[555]. However, a TTE was necessitated by technical limitations of the equipment and software setup available.

Whilst 60 g/hr is considered optimal when provisioning glucose, or glucose polymers, during prolonged exercise, the gold standard approach would include an additional 30 g/hr as fructose[144,243,339,382,556]. This was not possible here though, due to the prohibitive cost of U-^{13}C-fructose.

Furthermore, during exogenous carbohydrate oxidation calculations, background ^{13}C enrichment was taken at the fasted timepoint which carries a risk of overestimating oxidation rates[469]. Best practice would have been to replicate Experimental visits[185,478] with low/unenriched maltodextrin[473,479] and no U-^{13}C-glucose to establish backgrounds at each timepoint, however this would have doubled participant burden so was not deemed viable. Instead background ^{13}C enrichment was minimised and carbohydrate drinks were highly enriched, diminishing the relative impact of any background shifts[185]. Nonetheless, as adipose tissue was unlikely to have been depleted of ^{13}C across the pre-visit diet, and adipose lipolysis appeared to differ greatly between conditions, it cannot be discounted that oxidation of liberated FAs might have differentially influenced $^{13}CO_2$ appearance.

Additionally, the oxidative fates of the maltodextrin and U-^{13}C-glucose were assumed to be alike, as digestion of maltodextrin into glucose is not considered the rate-limiting step for absorption[466,557]. Evidence for this equivalency can be found in Hawley et al.'s Letter to the Editor[558] concerning their

1991 paper[559], alongside findings of glucose and glucose polymers being oxidised at the same rate[560–563], and a glucose-maltodextrin combination (or similar) having been employed previously[240,557,559,564,565]. However, it is possible that the acidity of βHB liberated from the KME could alter amylase and maltase activities, thus maltodextrin digestion rates[466]. It was also not possible to confirm the assumption that ^{13}C was randomly distributed across maltodextrin carbons[566].

4.4.9 Conclusions

It has been suggested that ketone oxidation might improve exercise economy, a key endurance performance determinant. However, observations presented here suggest that exogenous ketosis may either not affect, or worsen, the resilience of economy, during moderate intensity cycling in a low-carbohydrate setting where ketolytic capacity is posited to be greatest. This may be consequent of impaired SM contractile efficiency, thus increased type II fibre recruitment, though further exploration of the mechanisms underpinning these findings is required.

Chapter 5 - Modulation of Hepatic and Circulating Metabolism by Exogenous Ketones in a Fed and Fasted State

5.1 Introduction

Alongside ongoing interest in the ketone monoester (KME) for enhancing endurance exercise performance, its potential application as an adjuvant therapy for metabolic disorders[567–569] is gaining increased attention, for example as a nutritional intervention to lower blood glucose in those with type 2 diabetes (T2D)[158,160,161,329,332,570]. However, metabolic diseases such as T2D are multidimensional and extend beyond aberrant glycaemic control, with lipid metabolism playing a role in pathogenesis[320]. Fatty acid (FA) metabolism in the liver, for example, is a key regulator of whole-body insulin resistance and glucose metabolism[298] with ectopic accumulation of intra-hepatic triacylglycerol (IHTAG) not only a risk factor for fatty liver conditions such as MASLD (previously NAFLD[571]), but also for T2D and cardiovascular disease[319,331,572,573]. As chronically upregulated *de novo* lipogenesis (DNL) is suggested to be a driver of pathological IHTAG accretion[573], understanding how it might be modulated by exogenous ketosis would be valuable for characterising the KME's prospective capacity as a therapeutic. Whilst rodent studies have observed repeated exogenous ketone ingestion to slow the onset of hepatic steatosis, suggested within this work to be potentially driven by lowered lipogenesis[574,575], the impact of ketosis on DNL has not yet been directly assessed.

Whether taken for exercise or therapeutic purposes, an advantage that the KME carries over other exogenous ketone supplements (EKS) is its ability to safely elevate circulating βHB to >2 mM[31,40], with ketone levels appearing to influence metabolism[37,105], as well as potentially exercise ergogenicity[24], in a dose-dependent manner, as explored in *Chapter* 3. To better inform KME ingestion timing and dosage

recommendations, there is therefore a need to more fully understand how the time course of subsequent ketosis might be influenced by external factors, such as feeding state. Despite post-KME ingestion βHB kinetics being well described when fasted[20,23,110,576], how prior feeding might impact ketotic profiles remains poorly characterised. When comparing studies where the KME was consumed in a fasted (postabsorptive) state[20,23,229,230,239,241,70,71,107,110,136,153,159,162] to findings, including in *Chapters 3 & 4*, where the KME was ingested when fed (postprandial)[94,125,233,237,146,150–152,154,193,231,232], it appears that prior/concurrent ingestion of other macronutrients, carbohydrate in particular, might slow the rate of appearance, and lower achievable concentrations, of βHB in circulation. As a major user-population of the KME is endurance athletes[30,228], a logical setting in which to describe circulating βHB profiles, and the metabolic impact of this ketosis, would be after a substantial high-carbohydrate meal, akin to one consumed pre-training/race[3,5].

This study therefore carried two aims:

i. To explore how exogenous ketosis might modulate hepatic DNL in humans, and
ii. To characterise how plasma βHB profiles, and changes to wider metabolism consequent of this ketosis, might differ post-KME ingestion in fasted and fed states.

5.2 Methods

5.2.1 Study Design

This was a single-blind, semi-randomised, and counterbalanced, repeated measures study, registered at *www.clinicaltrials.gov* (NCT06320522). Participants completed a Baseline testing visit followed by four or five Experimental testing visits (*Figure 5.1*). So that findings would not be impacted by cofounders of pathological-state or exercise[257,366,367], as this was principally exploratory rather than iterative work, a population free from metabolic disease was studied, with participants at rest throughout.

During the Baseline visit, which took place within two weeks prior to the first Experimental visit, participants were enrolled into the study and anthropometric measures were taken. Subsequently, across the first four Experimental visits, participants either ingested a standardised high-carbohydrate breakfast (FED) or remained fasted (FAST), then consumed a drink containing either the KME or a taste and volume matched non-caloric placebo (PLA), after which they sat at rest for six hours whilst data was collected. Visit combinations were therefore fasted with the KME (FAST-KME) or with the placebo (FAST-PLA), and fed with the KME (FED-KME) or with the placebo (FED-PLA). A subset of participants returned for a fifth Experimental visit which matched the KME-FED protocol, except KME ingestion preceded, rather than proceeded, the breakfast (FED-REV).

Experimental visits were separated by at least a week, with participants consuming the same prescribed high-carbohydrate high-sugar diet for the two preceding days. The first and fourth visits were both FED to ensure a ≥21 day washout period[577] between them, as D_2O (heavy water) was provided. Most participants completed the first four Experimental visits in 3-5 weeks, within a maximum period of 6 weeks. The fifth visit (KME-REV) was exploratory post-hoc work to investigate KME-meal order effects[416,578], and took place within six months of the fourth Experimental visit.

A randomly generated matrix was used to ensure all possible condition orders were equally represented across Experimental visits 1-4 to minimise the chance of order-effects, with participants assigned a predetermined order based on enrolment date.

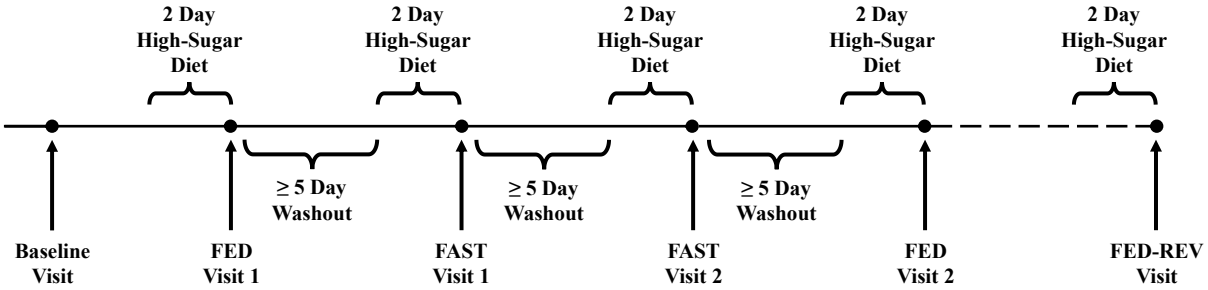


***Figure 5.1* - Overview of study timeline.**

FAST, postabsorptive; FED, postprandial with KME/PLA consumed post-meal; FED-REV, postprandial with KME consumed pre-meal.

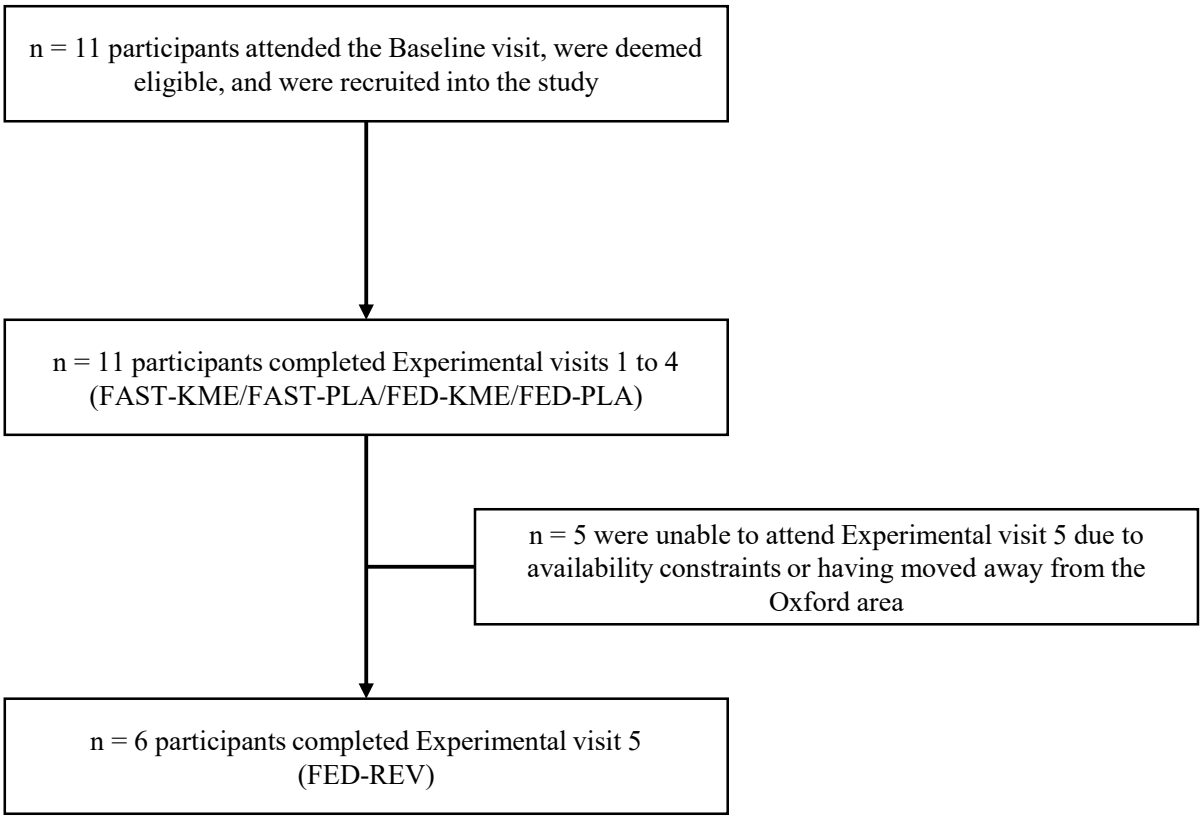


***Figure 5.2* - Study CONSORT diagram.**

Participants

Eleven participants were recruited from the local Oxford area (*Figure 5.2*), with inclusion criteria found in 2.2. Participant characteristics are summarised in *Table 5.1*. Six of these participants completed the additional FED-REV visit, with characteristics of this six only summarised in *Table 5.2*.

***Table 5.1* - Participant characteristics (n = 11).**

Gender	F/M	6	/	5
Age	yr	28.9	±	2.2
Height	cm	175.9	±	4.3
Weight	kg	74.8	±	5.8
BMI	kg/m^2	24.3	±	1.1

All study participants. BMI, Body Mass Index; F, female; M, male. Data presented as Mean ± SEM.

***Table 5.2* - Participant characteristics (n = 6 who additionally attended the FED-REV visit).**

Gender	F/M	3	/	3
Age	yr	25.7	±	1.7
Height	cm	175.5	±	5.3
Weight	kg	77.2	±	7.7
BMI	kg/m^2	24.9	±	1.6

Subset of participants who attended the additional fifth Experimental visit (FED-REV). BMI, Body Mass Index; F, female; M, male. Data presented as Mean ± SEM.

5.2.2 Baseline Visit

Participants were screened and enrolled into the study as detailed in 2.3.2.

5.2.3 Prior to Each Experimental Visit

Prior to each Experiment visit, participants refrained from alcohol (48 hr), caffeine (12 hr), and strenuous exercise (24 hr), as per 2.3.1. In addition, they consumed an eucaloric high-carbohydrate high-sugar prescribed diet for the two preceding days. This diet was delivered directly to participants and matched their estimated daily kcal requirements (2656 ± 167 kcal/day), with adherence confirmed verbally at each visit. Resting energy expenditure (REE) was calculated using Mifflin-St Jeor equations[579] (*Equation 5.1*), with the thermic effect of feeding assumed at 10%[580], and an activity factor[581] applied based on participants' reported weekly physical activity levels (*Table 5.3*). The target diet composition was calorically split 60:20:20 carbohydrate:protein:fat, with 55% of carbohydrate coming as free sugars. Fat was assumed to contain 9 kcal/g, with carbohydrate and protein as 4 kcal/g. The exact daily

macronutrients provided can be found in *Table 5.4*, with 54.9 ± 2.4% of carbohydrate being free sugars. An example diet is found in Appendix M.

As hepatic DNL would be expected to be low in the young healthy individuals studied[302], and the magnitude and directionality (i.e. up or down-regulating) of exogenous ketosis' influence on lipogenesis was unknown, a diet high in sugar was chosen to upregulate DNL pathways[317,582]. This would then provide greater scope for modulation by the KME, improving the resolution of findings from this exploratory work. Additionally, this diet aligned with recommendations of high-carbohydrate high-glycaemic index feeding for endurance athletes to optimise post-exercise glycogen resynthesis and during 'carbohydrate loading'[3,383,583–585].

***Equation 5.1* - Estimated resting energy expenditure.**

$$Resting\ Energy\ Expenditure = 10 \times W + 6.25 \times H - 5 \times A + x$$

A, age (yr); H, height (cm); W, weight (kg). For males: x = 5. For females: x = -161.

***Table 5.3* - Activity factors applied to resting energy expenditure.**

REE	Activity Factor	Definition
x 1.2	Sedentary	Little to no exercise
x 1.375	Light Activity	Light exercise/sports 1-3 days per week
x 1.55	Moderate Activity	Moderate exercise/sports 3-5 days per week
x 1.725	Very Active	Hard exercise/sports 6-7 days per week
x 1.9	Extra Active	Very hard exercise/sports and physical job

REE, resting energy expenditure.

***Table 5.4* - Two day prescribed diet macronutrient composition.**

Total	kcal	2599	±	162
Carbohydrate	kcal	1561	±	125
	%	60.5	±	3.2
Fat	kcal	511	±	31
	%	19.9	±	0.9
Protein	kcal	528	±	118
	%	19.6	±	3.2

%, percentage of total kcal from that macronutrient. Data presented as Mean ± SEM per day.

D_2O Provision

Participants consumed D_2O (99.8%, DLM-2259-1, Cambridge Isotope Labs, MA, USA) the evening prior to, and during, the FED-KME and FED-PLA visits to facilitate quantification of postprandial DNL as deuterium incorporation into newly hepatically synthesised palmitate, the gold standard methodology[577,582,586–588]. A 'loading' dose of D_2O was provided at 4 g/kg of body water (209.6 ± 16.4 g; body water estimated to be 70% of bodyweight[589]) with a target plasma enrichment of 0.4%[577]. Participants consumed this 10-12 hr before each visit, split into two doses taken 2 hr apart to decrease the likelihood of 'dizziness'[577]. From the point of having drunk the first half of the loading dose until the end of the subsequent day's visit, participants consumed filtered water enriched with D_2O at 0.4% *ad libitum*, to maintain consistent plasma enrichment. Participants collected a urine sample prior to ingesting any D_2O, from which background enrichment was quantified.

5.2.4 Experimental Visits

Menstrual Cycle Control

Three of the six self-identified female participants were not on any form of contraceptive, whilst two reported taking the combined pill (ethinyl oestradiol/levonorgestrel) and one as having a hormonal coil fitted. Substrate metabolism is known to vary across the menstrual cycle[590–592] , with DNL potentially ~three-fold elevated in the follicular phase compared to the luteal phase[593–596]. Therefore all visits for participants with menstrual cycles were timed to likely fall within the luteal phase[346,347]. As steroid hormones levels were not assessed, this was assumed to occur 14-28 days after onset of menstruation[345,348], adapted based on participant feedback.

Experimental Visit Protocol

Participants undertook four or five Experimental testing visits, with the protocols only differing in whether they ate a standardised breakfast or not, whether they consumed KME or PLA drinks, and in which order these took place. The protocols are described in *Figure 5.3* and *Figure 5.4*.

Postprandial (FED)	FASTED	-1 hr	0 min		15 min	30 min	45 min	1 hr	1.5 hr	2 hr	2.5 hr	3 hr	4 hr	5 hr	6 hr
Blood	X	*Std. Breakfast*	X	*KME/PLA*	X	X	X	X	X	X	X	X	X	X	X
Breath Acetone	X		X		X	X	X	X	X	X	X	X	X	X	X
DNL	X											X			X
Indirect Calorimetry	X							X						X	
Appetite & GI Scales	X		X			X		X		X		X	X	X	X
Urine			*Throughout*												

Postprandial Reverse Order (FED-REV)	FASTED	-2 hr	-1 hr 15 min to -1 hr	0 min	15 min	30 min	45 min	1 hr	1.5 hr	2 hr	2.5 hr	3 hr	4 hr	5 hr	6 hr
Blood	X	*KME*	*Std. Breakfast*	X	X	X	X	X	X	X	X	X	X	X	X
Breath Acetone	X			X	X	X	X	X	X	X	X	X	X	X	X
Indirect Calorimetry	X							X						X	
Appetite & GI Scales	X			X		X		X		X		X	X	X	X
Urine		*Throughout*													

Fasted (FAST)	0 min		15 min	30 min	45 min	1 hr	1.5 hr	2 hr	2.5 hr	3 hr	4 hr	5 hr	6 hr
Blood	X	*KME/PLA*	X	X	X	X	X	X	X	X	X	X	X
Breath Acetone	X		X	X	X	X	X	X	X	X	X	X	X
Indirect Calorimetry	X					X						X	
Appetite & GI Scales	X			X		X		X		X	X	X	X
Urine	*Throughout*												

***Figure 5.3* - Experimental visit sampling timelines.**

X, measure collected at that timepoint. Indirect Calorimetry (respiratory gas exchange) data collected prior to 0 min during the FAST protocol, otherwise collection period started at the given timepoint. DNL, de novo lipogenesis quantified. GI, Gastrointestinal; KME, ketone monoester; PLA, placebo; Std., Standardised.

Upon arrival, participants were weighed and cannulated, fasted blood samples were obtained, then respiratory gas exchange (indirect calorimetry) data was collected. As detailed in *Figure 5.4*:

- During FAST visits, participants immediately then consumed the KME or PLA drink,
- During FED visits, participants then ate a standardised breakfast, sat at rest for 1 hr, before consuming either the KME or PLA drink, and
- During FED-REV visits, participants then consumed the KME or PLA drink, sat at rest for 45 min, before eating the breakfast over the subsequent 15 min.

The 0 min timepoint was when the KME/PLA drink was ingested during FED and FAST visits, and 1 hr exactly after the breakfast had been finished for FED-REV. Therefore, for FED-KME, FED-PLA, and FED-REV visits, 0 min was 1 hr after the breakfast had been consumed. From 0 min onwards, the protocol for all Experimental visits was the same, with participants sat/reclining in a bed at rest for 6 hr.

As arrival times were kept consistent across all visits (2.3.4), KME/PLA ingestion occurred 1 hr, plus the time taken to eat the breakfast, later relative to arrival during the FED compared to the FAST visits. Whilst this 0 min timepoint could have been aligned to the same time of day for all visits, with arrival times remaining consistent to ensure the same wake-up time, this would have further increased participant burden by asking them to remain fasted for an increased duration during the FAST visits.

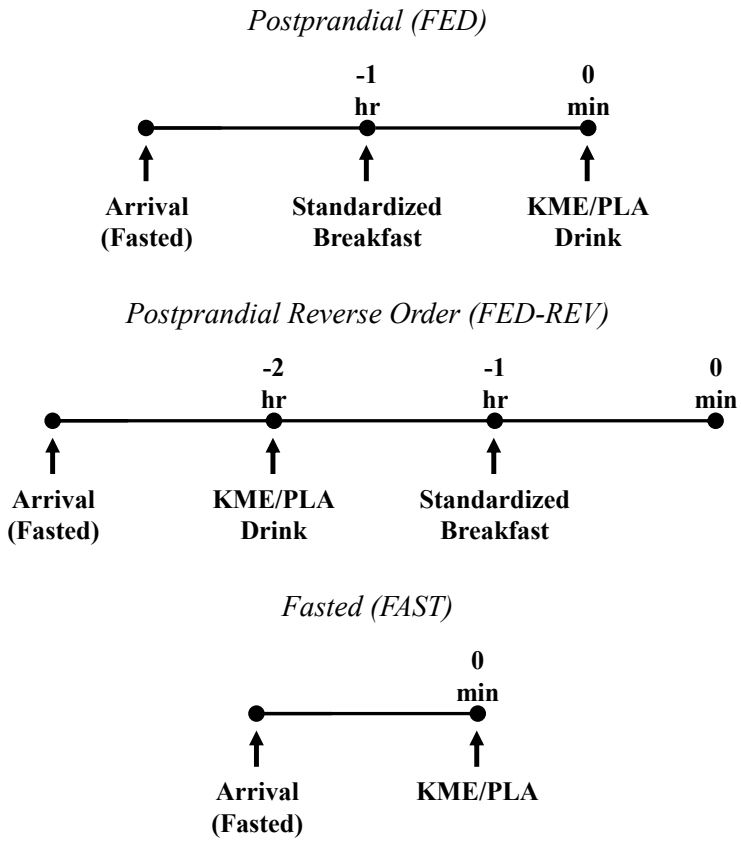


***Figure 5.4* - Experimental visit timeline from arrival to 0 min.**
From 0 min onwards, all visits follow the same protocol. KME, ketone monoester; PLA, placebo.

Standardised Breakfast

A standardised breakfast mixed-meal was consumed during FED-KME, FED-PLA, and FED-REV visits, providing 2 g/kg·BW (bodyweight) of carbohydrate as per 2.3.4 (exact macronutrients: *Table 5.5*). For the FED-KME and FED-PLA visits, the orange juice, and milk used to make porridge, were enriched with D_2O to 0.4%.

***Table 5.5* - Standardised breakfast macronutrient composition.**

Total Calories	kcal	941	±	88
Carbohydrate	g	149.8	±	11.5
Protein	g	23.6	±	2.6
Fat	g	25.6	±	3.8

Data presented as Mean ± SEM.

KME/Placebo Drinks

KME/PLA drinks were given at 0 min (FED and FAST) or -2 hr (FED-REV) and were prepared as per 2.3.3. KME was provided at 573 mg/kg·BW (42.9 ± 3.3 g; 206 ± 16 kcal), matching the initial dose given for the High-KME condition in *Chapter 3* and aligning with previous work[107,110,150,154,457]. For the FED-KME and FED-PLA visits, drinks were enriched at 0.4% with D_2O.

Drinks were prepared by the study investigator and thus condition was single-blinded for visits 1-4. It was not possible to blind the FED-REV visit as only one visit involved a reversed drink and breakfast consumption order, thus the nature of this protocol (i.e. the study drink being KME) was too easily deducible by participants for blinding to be effective.

5.2.5 Blood & Urine Sampling

Plasma Biochemistry

Blood samples were collected for plasma (***Figure 5.3***) as described in 2.3.4. Plasma samples from which the VLDL-rich fraction was to be isolated (3 mL) were kept on ice until the end of the visit, with 6 µL ethylenediaminetetraacetic acid (EDTA; 0.5 mol/L, pH 7.4), 3µL phenyl-methyl-sulphonyl-fluoride (PMSF; 10 mmol/L in propan-2-ol), and 15µL Trasylol (10,000 KIE/mL; 93482, Sigma Pharmaceuticals, Watford, UK) added to prevent sample degradation.

As per 2.4, plasma glucose, triacylglycerol (TAG), beta-hydroxybutyrate (βHB), non-esterified fatty acids (NEFA), lactate, glycerol, urea, total cholesterol, and high density lipoprotein (HDL)-cholesterol concentrations were analysed at all blood sampling timepoints. Additionally, TAG and Apolipoprotein B (ApoB; fasted timepoint only) concentrations were assessed for the VLDL-rich fraction (5.2.6).

βHB disposal rates under exogenous ketosis were estimated (2.4). Rates were only calculated for the FAST-KME visit as no tracer-derived KB oxidation data exists for a postprandial state[70,71].

Plasma insulin and C-peptide concentrations were determined using enzyme-linked immunosorbent assays (ELISA) kit (insulin: 10-1113-10, intra-assay <7.5%, sensitivity 6 pmol/L; C-peptide: 10-1136-01, intra-assay <6%, sensitivity <25 pmol/L; Mercodia, Uppsala, Sweden), per the manufacturer's instructions. Plates were read spectrophotometrically (FLUOstar Omega, BMG LABTECH Ltd, Aylesbury, UK). Insulin was assessed across all visits. C-peptide was assessed for the FED-KME and FED-PLA visits of two participants as exploratory work. These participants were chosen as those exhibiting the most positive and the most negative responses in DNL to exogenous ketosis compared to PLA, to investigate whether their contrasting responses were associated with differential insulin secretion (ISR) and clearance (ICR) rates. **The following calculations were performed by Dr Kieran Smith:** prehepatic ISR was calculated by utilizing a two-compartmental model for C-peptide kinetics and population-derived C-peptide parameters[597], alongside a single-pool model for ICR[598,599], incorporating participant characteristics, the nature of the breakfast mixed-meal, and plasma glucose concentrations, as previously described[578].

Urine

Urine was collected from when the first KME/PLA drink was given (FED/FAST, 0 min; FED-REV, -2 hr) until 6 hr after that point (FED/FAST, 6 hr; FED-REV, 4 hr), with participants requested to empty their blader at the end of this period. Total volume was recorded and a 2 ml sample collected for analysis of βHB and urea, as described in 2.4.

5.2.6 VLDL Isolation, TAG Extraction, and Quantification of DNL

VLDL Isolation

Immediately following FED-KME and FED-PLA visits, plasma chylomicron (Svedberg flotation rate [S_f] >400) and VLDL-rich fractions (S_f 20-400) were separated from samples taken at fasted, 180 min, and 360 min timepoints through sequential flotation using density gradient ultracentrifugation[600,601]. To achieve this, plasma was diluted in 1.5 mL NaBr (d1.42 kg/L) to achieve a plasma density of d1.10 kg/L. 4 mL of this d1.10 kg/L plasma was added to polyvinyl-alcohol (PVA) coated centrifuge tubes (14 x 95mm; #344060, Beckman Coulter, CA, USA). 3mL NaCl (d1.063 kg/L), 3mL NaCl (d.1.02 kg/L), and 2.8mL NaCl (d1.002 kg/L) were sequentially added, fed under gravity through a 16G needle touching the side of the tube, to create a three-layer NaCl gradient above the plasma. Samples were ultracentrifuged at 40000 rpm (285,000 G) and 15˚C for 32 min (rotor: SW40Ti; ultracentrifuge: Optima L-70, Beckman Coulter). The top 0.5-1 mL chylomicron fraction was aspirated into pre-weighted tubes and stored at -30°C but not analysed. 0.5-1mL NaCl (d1.006 kg/L) was added to centrifuge tubes to replace the volume aspirated, with samples further centrifuged for 16.5 hr (40000 rpm, 15˚C). The top 0.5-1mL was then aspirated into pre-weighted tubes, representing the VLDL-rich fraction.

Total Lipid Extraction

To establish the FA composition and enrichment of the VLDL-TAG plasma lipid pool, total lipids were extracted via the Folch method[602]. 500 µL of the isolated VLDL-rich plasma fraction was transferred into a glass vial. Internal standards of a known concentration (glyceryl triheptadecanoate [15:0; T2151-

100MG, Sigma Aldrich, MO, USA], heptadecanoic acid [17:0; H3500-1G, Sigma-Aldrich], and 1,2-diheptanoyl-sn-glycero-3-phosphocholine [17:0; P4148-100MG, Sigma-Aldrich]) were added to enable calculation of FA concentrations. Additionally, 5 mL chloroform:methanol (2:1 v/v) and 1 mL 1 M NaCl were added. Samples were vortexed and centrifuged (800 g, 5 min, 15˚C; J6, Beckman Coulter). The aqueous top phase was aspirated and discarded whilst the lipid-containing phase was completely dried under nitrogen at 50˚C in a water-bath evaporator (Zymark TurboVap LV Evaporator, Marshall Scientific, NH, USA).

Separation of TAG Fraction

The TAG lipid fraction was isolated from total lipids by solid-phase extraction (SPE) using ISOLUTE columns containing aminopropyl silica (470-0010-A, Biotage, Uppsala, Sweden). NEFA and phospholipid (PL) fractions were first separated from TAG and cholesterol-ester (CE) fractions. Columns were prewashed (twice with acetone, twice with chloroform). The total lipid sample was reconstituted in 1 mL chloroform then added to the column under gravity. NEFA and PL were retained within columns, with TAG and CE passing through. Columns were washed twice with chloroform under vacuum then discarded, with the collected TAG-CE fraction dried completely under nitrogen at 50˚C. The TAG-CE fraction was reconstituted in 1 mL hexane, and fresh columns were prewashed four times with hexane. Samples were added to the column under gravity, then washed through twice with hexane under vacuum, with TAG retained in the column. Isolated TAG was eluted from the columns through washing twice with hexane:chloroform:ethyl acetate (100:5:5 v/v/v), then dried completely under nitrogen at 50˚C.

FAME Synthesis

FA-methyl esters (FAME) were synthesised from the isolated TAG fraction to liberate individual FAs, decrease lipid polarity, and reduce FA boiling points[603]. Samples were incubated for 1 hr with 400 µL butylated hydroxytoluene (BHT):toluene (1:10 w/v) and 800 µL 1.5% H_2SO_4:methanol (v/v) to form FAMEs. This reaction was then quenched with 2 mL of a neutralising solution (125 mM $KHCO_3$ & 125

mM K_2CO_3 in H_2O). FAMEs were extracted by adding 2 mL of cyclohexane, with samples vortexed and centrifuged (2000 rpm; 14°C, 5 min). The FAME-containing top phase was aspirated off and dried completely under nitrogen at 50˚C. Samples were then reconstituted in chloroform, with a known concentration of external standard added (methyl tricosonate, 23:0; T9900-1G, Sigma-Aldrich).

Fatty Acid Composition

FA composition (µmol/100 µmol total FAs) of the VLDL-TAG plasma fraction was determined by gas chromatography (GC; 7890B, Agilent, CA, USA) using a capillary column (30 m × 0.530 mm x 1.00 µm; DB-WAX, 125-7032, Agilent) with helium as the carrier gas (2.0 mL/min). Samples were injected at 100 µL using splitless injection with an initial oven temperature of 60°C. This was increased at 20°C/min to 200°C, held there for 12 min, then increased at 2°C/min to 220°C, held there for 2 min, then increased at 1°C/min to 230°C, and held there for 25 min.

FA peaks were identified by comparison of sample retention time to that of a commercially available standards of 31 FAME (FAME Mix, C4-C24, #18919-1AMP, Merck Life Science UK Ltd, Gillingham, UK). FA concentration was established by standardising the peak area to that of the internal standard of a known concentration. Peak areas for all FAs in a sample were added together, excluding the standards, with the percent of the total FA area calculated for each FA as molar percent (mol%). Concentrations of the following fatty acids are reported in this work[586]: myristic acid (14:0), palmitic acid (‘palmitate’; 16:0), palmitoleic acid (‘palmitoleate’;16:1n-7; hence referred to as 16:1), stearic acid (18:0), oleic acid (18:1n-9; hence referred to as 18:1), and linoleic acid (‘linoleate’; 18:2n-6; hence referred to as 18:2).

Plasma & Urine D_2O Enrichment

Urine, used to determine pre-D_2O background body water deuterium enrichment, and plasma D_2O enrichment were determined in duplicate using gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS; Finnigan GasBench II Thermo Fisher Scientific, Paisley, UK). Plasma samples, from FED-KME and FED-PLA visits, were assessed for enrichment at the same Fasted, 180 min, and 360 min timepoints as where DNL was quantified. Before each run, a H_3 factor test was

performed. This acted to correct for the contribution of H_3^+ species to ^2H detection in the ion source as H_2 pressures increased[604].

300µL urine and 10µL plasma were diluted with 200µL and 490µL deionised water, respectively, with Vienna Standard Mean Ocean Water (VSMOW) standards also prepared. A platinum catalyst (1091831; Thermo Fisher Scientific) was added to each sample before being flushed with 2% hydrogen in helium for 5 min then left to equilibrate for ≥5 hr. A sample of gas was then injected into the IRMS, with the $^2H/^1H$ ratio measured ten times against the internal IRMS VSMOW value[588]. D_2O enrichment of each sample was determined using the last nine $^2H/^1H$ vs VSMOW measurements, which were then Standard Light Antarctic Precipitation (SLAP)[605] corrected and averaged. These averages were normalised to the VSMOW standards within each run, the dilution factor, and background urine enrichment. The calculated δ‰ vs VSMOW values were then converted to a tracer-to-tracee ratio (TTR; $^2H/^1H$) using:

***Equation 5.2* - TTR.**

$$TTR = \left(\left[\frac{\delta‰}{1000}\right] + 1\right) \times 0.00015595$$

Hepatic DNL as 16:0 Deuterium Enrichment

Deuterium (^2H) enrichment of palmitate was assessed by GC-MS[606] (GC: 6890N, MS: 5973N, Agilent) using a capillary column (30m × 0.25µm × 0.32 mm; RTX-5, 10224, Restek Thames, Saunderton, UK) with helium as the carrier gas. FAME samples were injected using splitless injection with an injector temperature of 250°C. The initial oven temperature was 50°C, which was held for 1 min, before being increased by 25°C/min to 200°C, then increased by 10°C/min to 230°C, where it was held for 10 min. Dwell time was 100 ms. Retention times were validated against a standard included in each run (AOCS#6, RM-6, Restek Thames).

Selective ion monitoring was performed to detect palmitate-derived ions with a m/z of 270 (M+0), 271 (M+1). A TTR for a given sample was calculated by dividing peak areas of the tracer ion (M+1) by the parent ion (M+0), i.e. 271/270. The natural abundance of ^2H-palmitate, taken from non-deuterium

enriched lipoprotein standards from an in-lab stock, was subtracted from TTRs of each sample. The proportion of newly synthesised, thus hepatic DNL-derived, palmitate (16:0) in VLDL-TAG was determined as:

***Equation 5.3* - DNL, as % newly synthesised palmitate.**

$$\%\ \text{Newly Synthesised 16:0 in VLDL} - \text{TAG} = \frac{16{:}0\ \text{TTR}}{\text{Plasma Water TTR} \times 22} \times 100$$

16:0, palmitate. 16:0 TTR and plasma water TTR both background-corrected.

Plasma D_2O enrichment is multiplied by 22 here as 22 hydrogens on palmitate could be labelled during DNL[587].

5.2.7 Cardiorespiratory Measures

Respiratory gas exchange (indirect calorimetry) data was collected as detailed in 2.3.6 (*Figure 5.3*). In each instance, sampling continued until a steady state had been achieved for 5 min, with averages taken across this 5 min period. A steady state was determined as $\dot{V}O_2$ and $\dot{V}CO_2$ deviating by <10% across this period. If a steady state was not detected within 30 min, then collection ceased with the data retrospectively analysed and the time interval over which values were averaged shortened until a steady state within the above parameters was found (min. 3 min). Substrate oxidation calculation under exogenous ketosis was not possible[69] and therefore wasn't performed on any datasets.

Breath acetone was measured using a breath ketone monitoring system (Biosense, Readout Inc., MO, USA). Data are presented as arbitrary 'ACE' units (validated against 'NIST-certified gas standards' at R^2 = 0.998, per manufacturer's documentation). ACE units equated to: 0-4, *no/low ketones*; 5-14, *moderate ketosis*; 15-30, *advanced ketosis*; 31-39, *deep ketosis*; ≥40, *high ketosis*.

5.2.8 Subjective Measures

Gastrointestinal (GI) Distress and Appetite measures (*Figure 5.3*) were assessed as described in 2.3.4.

5.2.9 Blinding Efficacy

The first four Experimental visits were single-blinded to intervention (KME or PLA) drink composition, with the fifth visit (FED-REV) not blinded. Participants were asked to try and identify each of the study conditions after they had completed the first four visits to assess blinding effectiveness. They were requested to identify each visit based on 'the taste/palatability of the drink'. They graded each selection with a certainty rating on a linear scale from 0 (completely uncertain) to 10 (completely certain).

5.2.10 Data Analysis

Outcome Measures

The primary outcome measure for this study was postprandial hepatic DNL (FED-KME vs FED-PLA) as % newly synthesised 16:0 in VLDL-TAG. Secondary outcomes were circulating metabolite profiles (FED-KME vs FED-PLA, FAST-KME vs FAST-PLA, and FED-KME vs FED-REV [n = 6 exploratory sub-study]) and plasma βHB profiles (FED-KME vs FAST-KME).

Sample Size Calculation

As no comparable data was available for the influence of exogenous ketosis on postprandial hepatic DNL, a sample size calculation was performed in G*Power[380] using internal pilot data, where an effect size of $f = 0.98$ was estimated. With a significance level (α) of 0.05 and power ($1-\beta$) of 0.8 (2 conditions, 3 timepoints) it was expected that 8 participants would be required. This study therefore aimed to recruit 11 participants to account for attrition and overestimates of observed effects.

Dataset Sample Sizes

One participant is excluded from DNL analyses as they failed to consume the necessary D_2O the evening prior to one of the FED visits, thus this dataset, and accompanying VLDL-TAG FA composition, carries a n = 10. The remaining datasets are n = 11, with the exception of comparisons to FED-REV visits which are n = 6 as not all participants returned for this ad-hoc fifth Experimental visit (*Figure 5.2*). All n values are reported on applicable figures and tables. Plasma and urine urea concentrations were not determined for FED-REV visits due to unavailability of necessary analytical reagents, thus are excluded from any comparisons including this condition.

Area-Under-The-Curve

Area-under-the-curve (AUC) was calculated from 0 min until 6 hr for selected metrics as per 2.5.

Statistics

Statistics were performed as described in 2.5. Simple linear regressions were undertaken where appropriate between Δ*metric* values (KME values *minus* PLA values, or FED-KME *minus* FED-REV, as appropriate) between two metrics.

As the primary research question for this work centred around how hepatic DNL might differ between the FED-KME and FED-PLA conditions only, and as the study was not sufficiently powered for 2x2x12 (*KME/PLA* x *FED/FAST* x *time*) three-way repeated-measures ANOVAs to assess circulating metabolite profiles across the first four visits[607], this dataset was split into separate FED-KME vs FED-PLA (5.3.1) and FAST-KME and FAST-PLA (5.3.4) analyses. This allowed for the influence of exogeneous ketosis on metabolism, compared to a placebo control state, to be separately profiled in postprandial and fasted nutritional states.

As comparisons between FED-KME and the reversed-order FED-REV condition carried a significantly lower sample size, these were assessed separately. The FED-PLA condition was not included in these analyses as effect sizes established from the FED-KME vs FED-PLA dataset indicated that the n = 6

sample size here was insufficient to properly power 3x12 (*intervention drink* x *time*) two-way repeated measures ANOVAs[380,448,480]. Due to the low power nature, risk of order-effects, and incomplete blinding this sub-study should be considered post-hoc exploratory (i.e. pilot) work.

Plasma βHB concentrations, in isolation, were evaluated between FED-KME vs FAST-KME to explore how nutritional state might affect βHB profiles after ingestion of the KME.

5.3 Results

5.3.1 Postprandial Visits (FED-KME vs FED-PLA)

Postprandial visits were compared to assess the impact of exogenous ketosis on hepatic DNL, as well as wider circulating metabolites, after consumption of a high-carbohydrate mixed-meal breakfast.

Plasma Biochemistry

Concentrations of all plasma metabolites and breath acetone were similar between conditions when overnight fasted at commencement of each visit (Appendix N).

Plasma βHB concentration exhibited effects of time and condition, alongside a time-x-condition interaction ($p < 0.001$; *Figure 5.5-A*). Accordingly, there was an effect of condition on βHB-AUC ($p < 0.001$; *Figure 5.5-B*) reflecting greater total circulating βHB exposure under FED-KME compared to FED-PLA. ΔβHB-AUC (Δ represents FED-KME-AUC *minus* FED-PLA-AUC for a given metabolite) was not predicted by age, height, weight, or BMI ($p \geq 0.134$).

Whilst circulating glucose levels did not change over time ($p = 0.252$; *Figure 5.5-C*), significant condition and interaction effects were observed ($p \leq 0.009$), with concentrations and glucose-AUC ($p < 0.001$; *Figure 5.5-D*) lower for FED-KME.

Effects of condition and time were observed for plasma insulin ($p \leq 0.007$; *Figure 5.5-E*) with levels elevated under FED-KME, though no interaction effect was present ($p = 0.190$). Insulin-AUC was commensurately greater during FED-KME compared to FED-PLA ($p = 0.005$; *Figure 5.5-F*). Exploratory n = 2 analysis to model insulin kinetics was undertaken for the participant with the most elevated DNL response during FED-KME ('positive' responder) relative to FED-PLA, and for the participant with the most supressed response ('negative' responder). For the positive responder, ISR-AUC was 39.11% greater across FED-KME relative to FED-PLA, compared to 60.00% for the negative responder. In contrast, ICR-AUC was 22.09% and 20.09% less during FED-KME for the positive and negative responders, respectively.

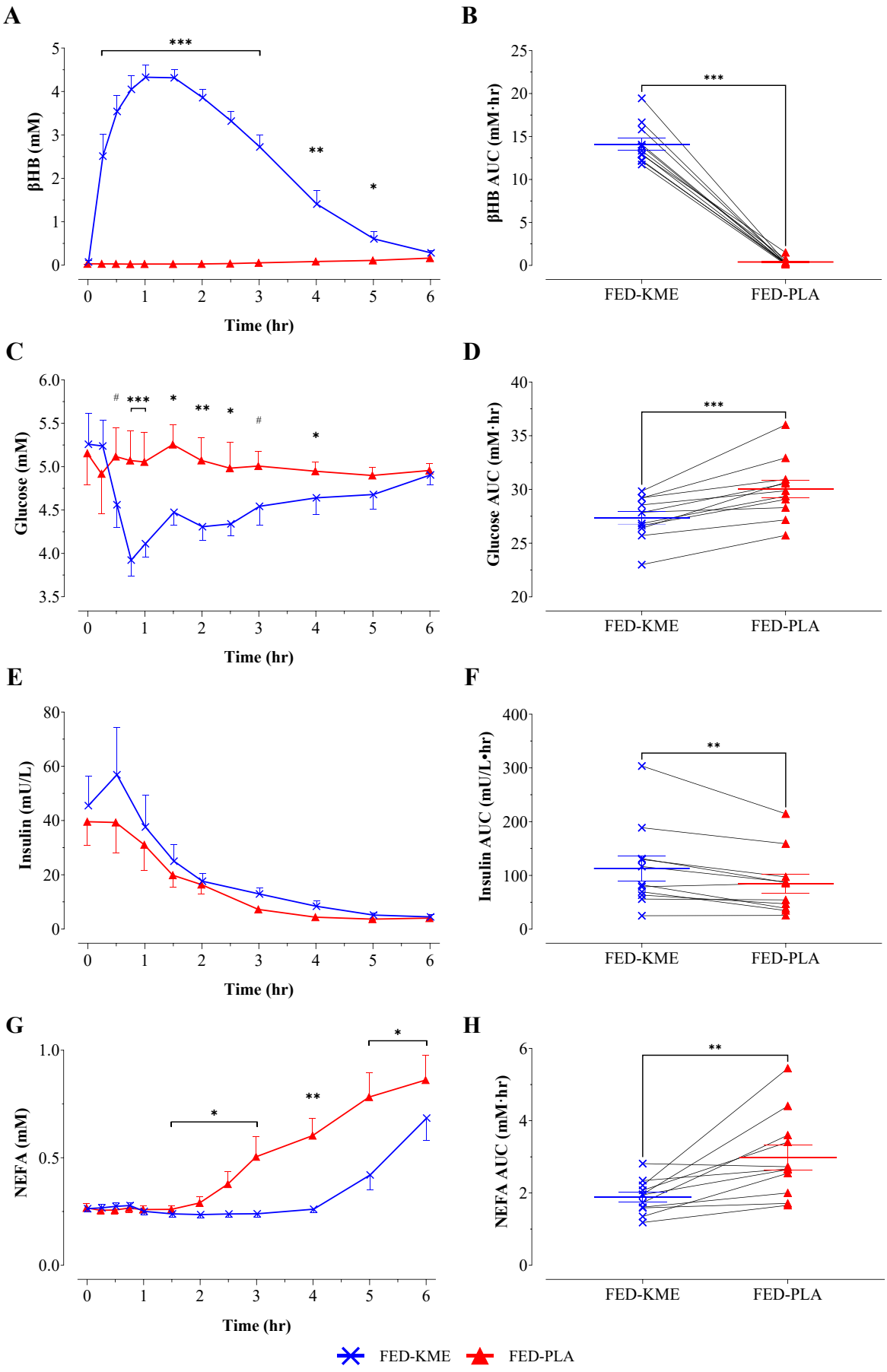


***Figure 5.5* - Plasma βHB, glucose, insulin, & NEFA during the FED-KME & FED-PLA visits.**

*A, βHB concentration (mM); **B**, βHB AUC (mM·hr); **C**, glucose concentration (mM); **D**, glucose AUC (mM·hr); **E**, insulin concentration (mU/L); **F**, insulin AUC (mU/L·hr); **G**, NEFA concentration (mM);*

***H**, NEFA AUC (mM·hr). AUC, area-under-the-curve. Data presented as Mean ± SEM (with individual values plotted for B/D/F/H). # $p < 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ between FED-KME and FED-PLA conditions. n = 11.*

Plasma NEFA displayed time, condition, and interaction effects ($p \leq 0.008$; *Figure 5.5-G*), whilst an effect of condition was present for NEFA-AUC ($p = 0.006$; *Figure 5.5-H*), with concentrations lower under FED-KME. Changes to circulating glycerol levels mirrored those found for NEFA, with time, condition, and interaction effects established ($p \leq 0.008$; Appendix N) and well as a condition effect for glycerol-AUC being lower under FED-KME ($p = 0.018$; Appendix N).

No effect of condition was found for plasma lactate or urea concentrations ($p \geq 0.285$; *Figure 5.6-A/B*), nor AUCs ($p \geq 0.230$), but time and interaction effects were present ($p \leq 0.047$). Neither condition ($p \geq 0.538$) nor interaction ($p \geq 0.148$) effects were established for plasma TAG (*Figure 5.6-C*), HDL-cholesterol, non-HDL-cholesterol, or total cholesterol concentrations (Appendix N; non-HDL-cholesterol data not presented), with AUCs accordingly not differing between conditions ($p \geq 0.442$). No time effect was present for HDL-cholesterol ($p = 0.222$), though a trend for an effect was observed for TAG ($p = 0.051$), whilst total cholesterol and non-HDL-cholesterol both changed over time ($p \leq 0.041$). Breath acetone exhibited time, condition, and interaction effects ($p < 0.001$; *Figure 5.6-D*), with both concentrations and acetone-AUC ($p < 0.001$) greater for FED-KME.

ΔβHB-AUC significantly positively predicted Δinsulin-AUC (*Figure 5.7-A*) and Δlactate-AUC ($R^2 = 0.446$; $p = 0.025$), but not ΔAUCs for any other metabolites ($p \geq 0.534$). Δinsulin-AUC and Δlactate-AUC positively predicted each other (*Figure 5.7-B*), with ΔNEFA-AUC and Δglycerol-AUC also being positively related ($R^2 = 0.920$; $p < 0.001$).

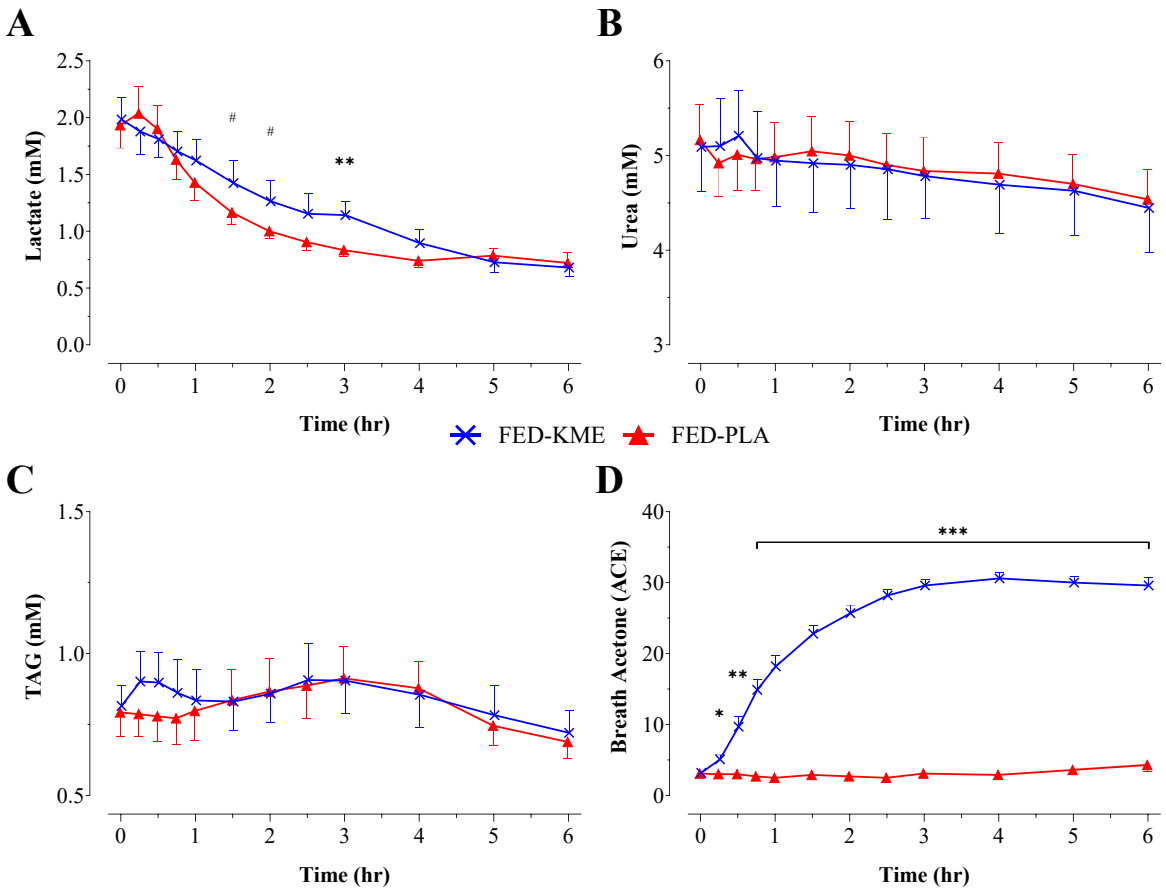


***Figure 5.6* - Plasma lactate, plasma urea, plasma TAG, & breath acetone concentrations during the FED-KME & FED-PLA visits.**

*A, plasma lactate (mM); **B**, plasma urea (mM); **C**, plasma TAG (mM); **D**, breath acetone (ACE; arbitrary units). Data presented as Mean ± SEM. # $p < 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ between FED-KME and FED-PLA conditions. n = 11.*

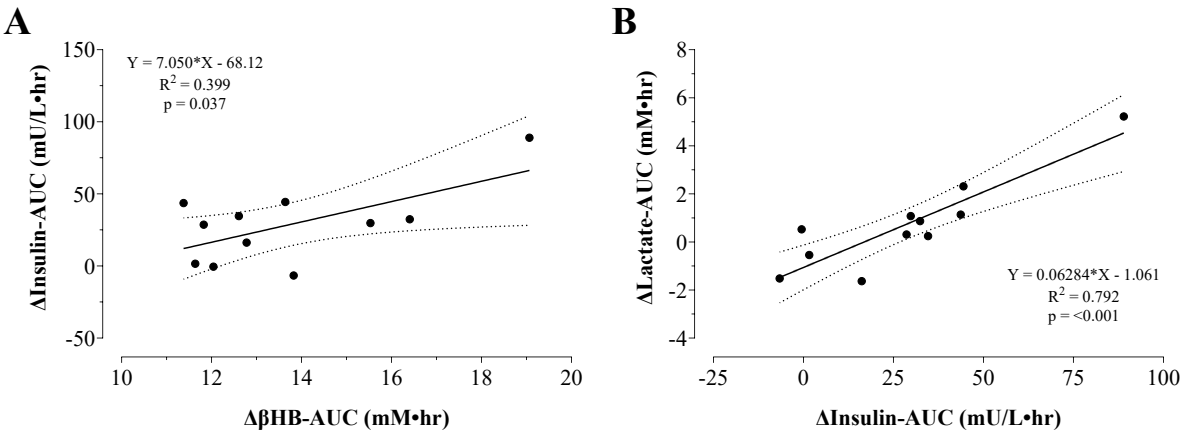


***Figure 5.7* - ΔβHB-AUC vs ΔInsulin-AUC & ΔInsulin-AUC vs ΔLactate-AUC across the FED-KME & FED-PLA visits.**

Δ, between condition difference calculated as FED-KME minus FED-PLA; AUC, area-under-the-curve. Solid line, linear best fit; dashed lines, 95% confidence intervals. n = 11.

Hepatic DNL & VLDL-TAG Composition

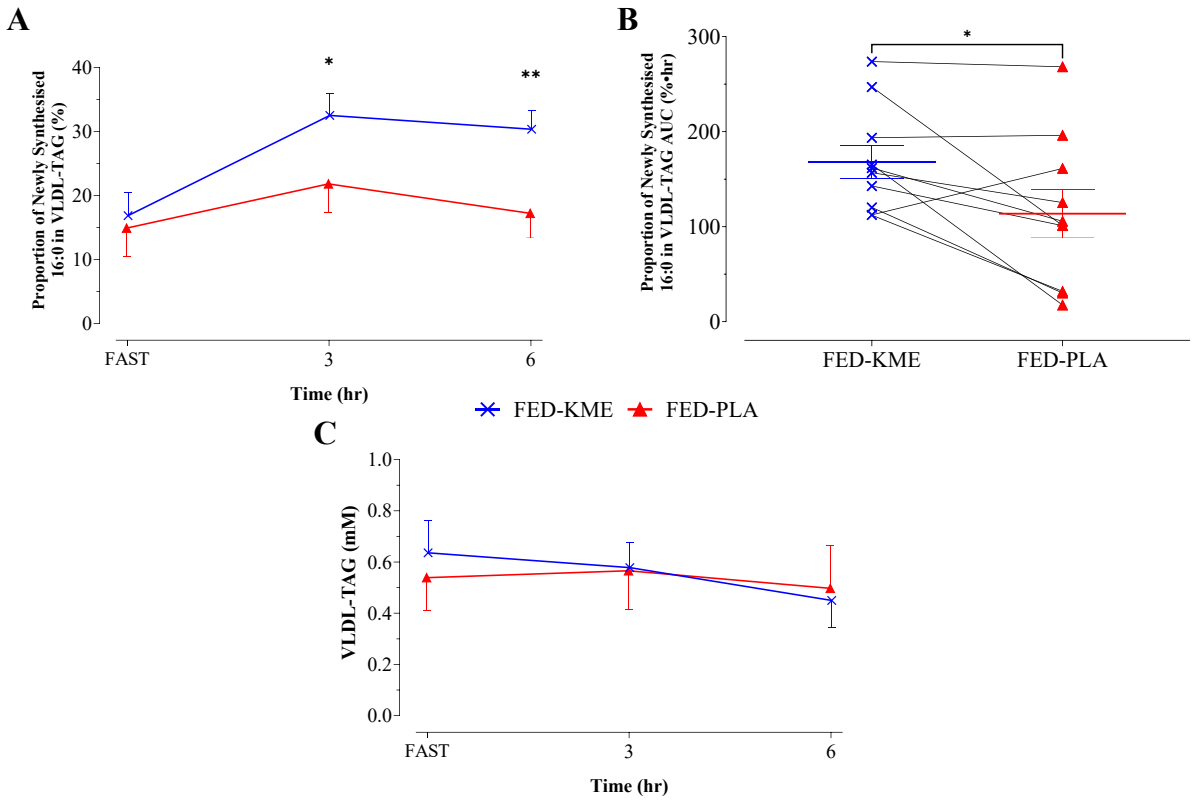


***Figure 5.8* - DNL & VLDL-TAG concentration across the FED-KME & FED-PLA visits.**
***A**, DNL (%); **B**, DNL AUC (%·hr); **C**, VLDL-TAG concentration (mM). DNL, de novo lipogenesis assessed as the proportion of newly synthesised palmitate (16:0) in VLDL-TAG. Data presented as Mean ± SEM (with individual values plotted for B). * $p < 0.05$, ** $p < 0.01$ between FED-KME and FED-PLA conditions. $n = 10$.*

DNL was assessed during FED-KME and FED-PLA visits as the proportion of newly synthesised palmitate (16:0) in VLDL-TAG. It carried time ($p < 0.001$; *Figure 5.8-A*), condition ($p = 0.025$), and interaction ($p = 0.018$) effects with DNL elevated across FED-KME compared to FED-PLA, being 11% / 49% increased at 3 hr and 13% / 76% increased at 6 hr (absolute / relative % difference). Reflecting this, DNL-AUC was greater in FED-KME compared to FED-PLA ($p = 0.020$; *Figure 5.8-B*). ΔDNL-AUC was not predicted by ΔAUCs for any circulating metabolite ($p \geq 0.292$).

VLDL-TAG concentration displayed an effect of time ($p = 0.037$; *Figure 5.8-C*), but not condition or interaction effects ($p \geq 0.232$). VLDL-ApoB concentration ($p = 0.288$), and the ratio of VLDL-TAG to VLDL-ApoB ($p = 0.658$), did not differ between conditions when overnight fasted.

No condition effects were exhibited for any FA concentrations ($p \geq 0.174$; Appendix N), assessed as a mol% of the total VLDL-TAG composition, whilst interactions effects were absent for 14:0, 16:1, 18:1, and 18:2 ($p \geq 0.174$). Trends for interaction effects were observed for 16:0 and 18:0 ($p \leq 0.066$), with exploratory post-hoc analyses undertaken for both measures based on these trends. Effects of time were found for all FAs ($p \leq 0.048$), except for 18:2 ($p = 0.963$).

Urine Biochemistry

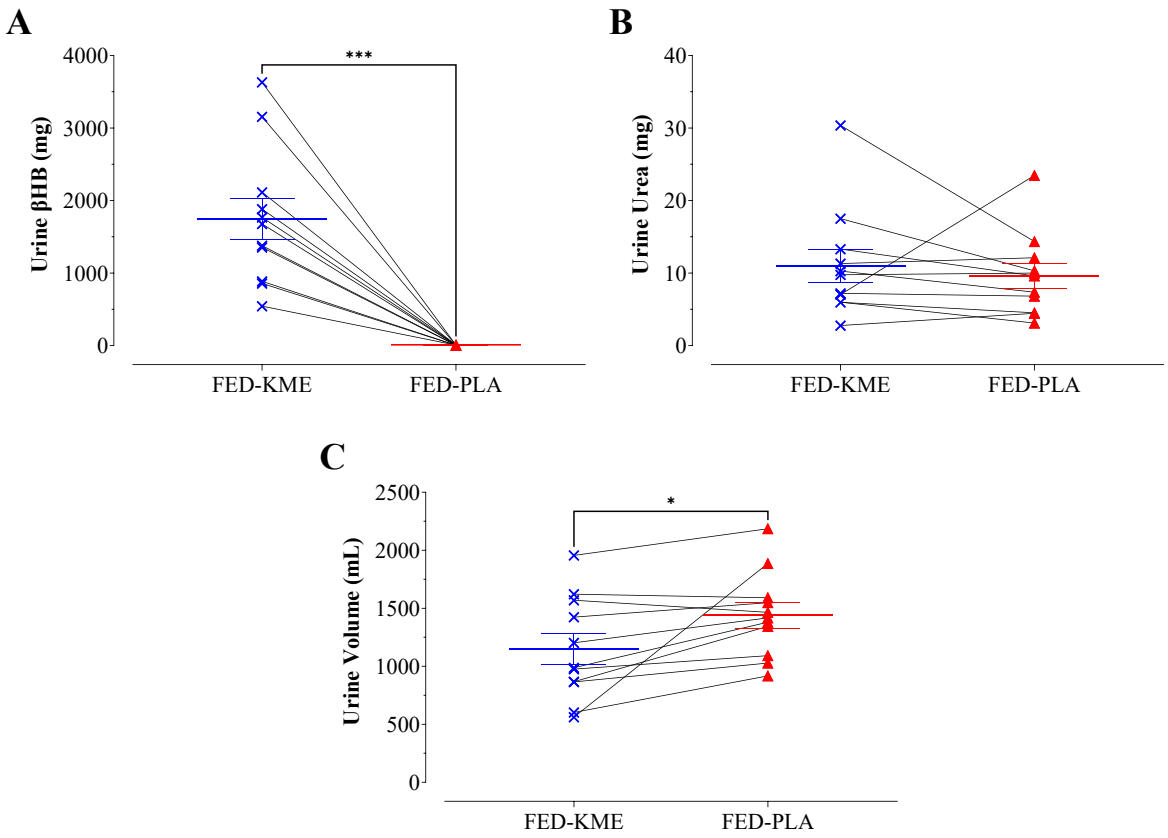


***Figure 5.9* - Urine metabolite excretion & volume across the FED-KME & FED-PLA visits.**
*A, urine βHB (mg); B, urine urea (mg); C, urine volume (mL). Urine collected from 0 min to 6 hr. Data presented as Mean ± SEM with individual values plotted. * $p < 0.05$, *** $p < 0.001$ between FED-KME and FED-PLA conditions. n = 11.*

Total βHB mass excreted in urine across each visit was greater during FED-KME compared to FED-PLA ($p < 0.001$; *Figure 5.9-A*), whilst total visit urine volume was lower under FED-KME ($p = 0.029$; *Figure 5.9-C*), and excreted total urea mass did not differ between conditions ($p = 0.560$; *Figure 5.9-B*).

Respiratory, Appetite, & GI Distress Measures

Effects of time but not of condition, nor interaction effects, were observed for $\dot{V}O_2$, $\dot{V}CO_2$, RER, BF, V_T, and V_E (Appendix N).

All appetite scores were similar between conditions when overnight fasted upon arrival for each visit ($p \geq 0.437$). Time effects were established for all appetite metrics ($p < 0.001$; *Figure 5.10*). *Hunger* and *Fullness* presented interaction effects ($p \leq 0.028$; *Figure 5.10-A/B*) in the absence of effects of condition ($p \geq 0.159$). Trends, or weak trends, for condition effects were observed for *Satisfied* ($p = 0.072$; *Figure 5.10-C*), *How Much Could Eat* ($p = 0.056$; *Figure 5.10-D*), and *Total Appetite* ($p = 0.075$; *Figure 5.10-E*). Interaction effects were absent from *Satisfied*, and *How Much Could Eat*, though a trend for an interaction effect was present for *Total Appetite* ($p = 0.061$).

No time, condition, or interaction effects were established for GI distress ($p \geq 0.589$) with total symptom scores not elevated above 0.9 at any timepoint for either condition indicating effects were negligible, thus neither KME nor PLA appeared to cause material GI upset.

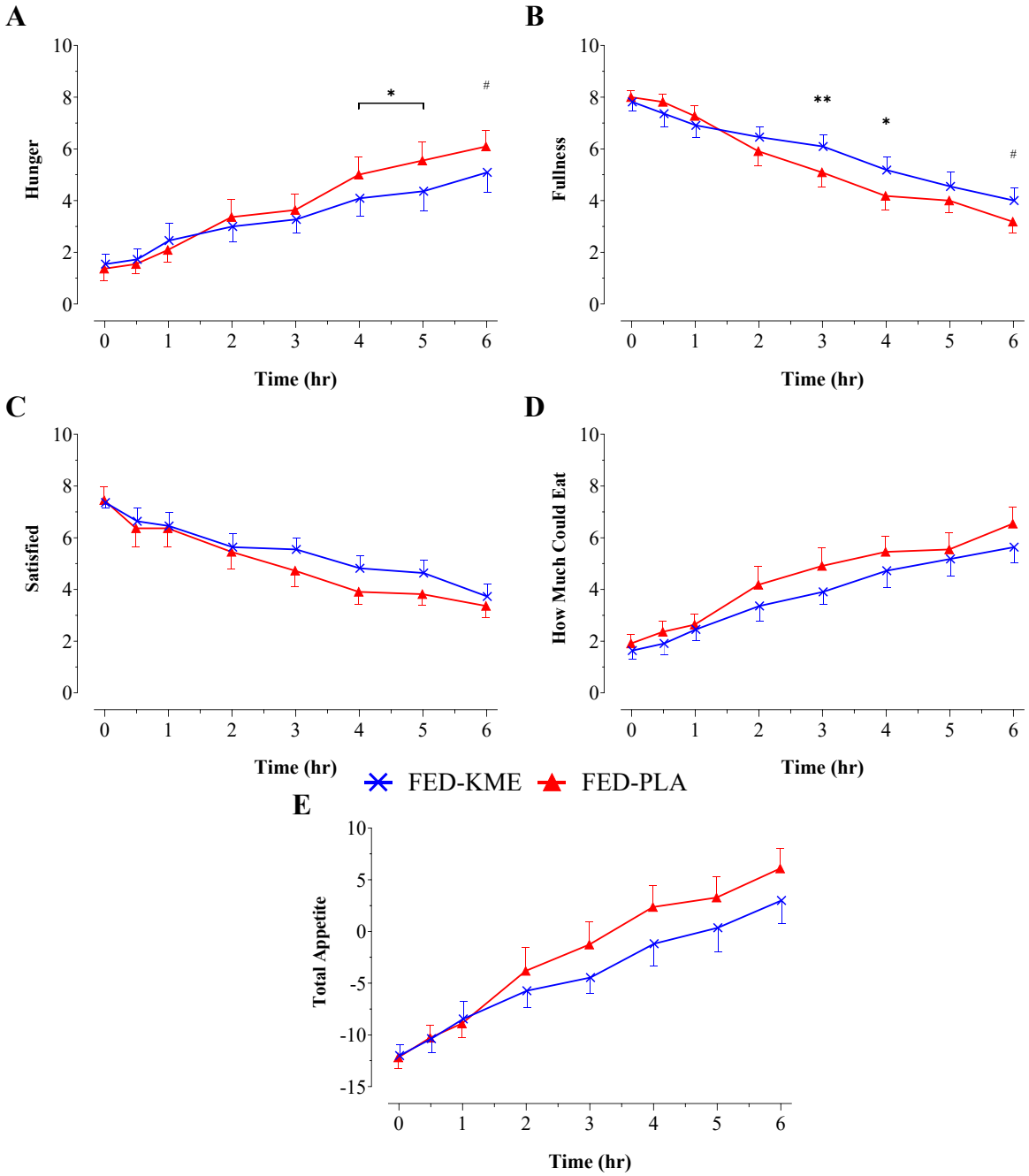


Figure 5.10* - *Appetite metrics across the FED-KME & FED-PLA visits.

***A,** Hunger; **B,** Fullness; **C,** Satisfied; **D,** How Much Could Eat; **E,** Total appetite, composite score of the four appetite metrics. Data presented as Mean ± SEM; 0-10 scale (A-D). $^{\#}$ $p < 0.05$, ***** $p < 0.05$, ****** $p < 0.01$ between FED-KME and FED-PLA conditions. n = 11.*

5.3.2 FED-KME vs FED-REV Visits

Exploratory pot-hoc analyses sought to establish if an order effect was present between consuming the KME 1 hr prior to a high-carbohydrate breakfast meal (FED-REV) compared to when it was consumed 1 hr post-meal (FED-KME).

Plasma Biochemistry

Concentrations of all plasma metabolites, insulin, and breath acetone were similar between conditions when participants were overnight fasted (Appendix O).

Plasma βHB displayed time, condition, and interaction effects ($p < 0.001$; *Figure 5.11-A*), with concentrations and βHB-AUC ($p < 0.001$; *Figure 5.11-B*) greater under FED-KME compared to FED-REV. Whilst no condition effect was observed for plasma glucose ($p = 0.133$; *Figure 5.11-C*), and consequently glucose-AUC did not differ between conditions ($p = 0.723$), time and interaction effects were established ($p \leq 0.006$). Circulating NEFA concentration exhibited time and interaction effects ($p \leq 0.022$; *Figure 5.11-D*), though no condition effect was present ($p = 0.614$), with NEFA-AUC not differing between conditions ($p = 0.438$). Similarly, time and interaction ($p < 0.001$; Appendix O), but not condition ($p = 0.687$), effects were present for plasma glycerol, though glycerol-AUC was greater for FED-REV ($p = 0.008$; Appendix O). An effect of time was present for plasma insulin ($p = 0.006$; *Figure 5.11-E*), though no condition or interaction effects were established ($p \geq 0.171$). However, a trend for an insulin-AUC condition effect was present ($p = 0.070$; *Figure 5.11-F*), indicating levels may have been lower under FED-REV.

Circulating lactate fell over time ($p < 0.001$; *Figure 5.12-A*), but no condition nor interaction effects was observed ($p \geq 0.0534$), with lactate-AUC not differing between conditions ($p = 0.960$). An interaction effect was established for plasma TAG ($p = 0.038$; *Figure 5.12-B*) alongside a weak trend for an effect of time ($p = 0.075$), though no condition effect was seen ($p = 0.993$) and TAG-AUC was similar between conditions ($p = 0.601$). Breath acetone exhibited time and interaction effects ($p < 0.001$; *Figure 5.12-C*), though no effect of condition ($p = 0.104$) was present, with acetone-AUC similar between FED-KME and FED-REV ($p = 0.190$).

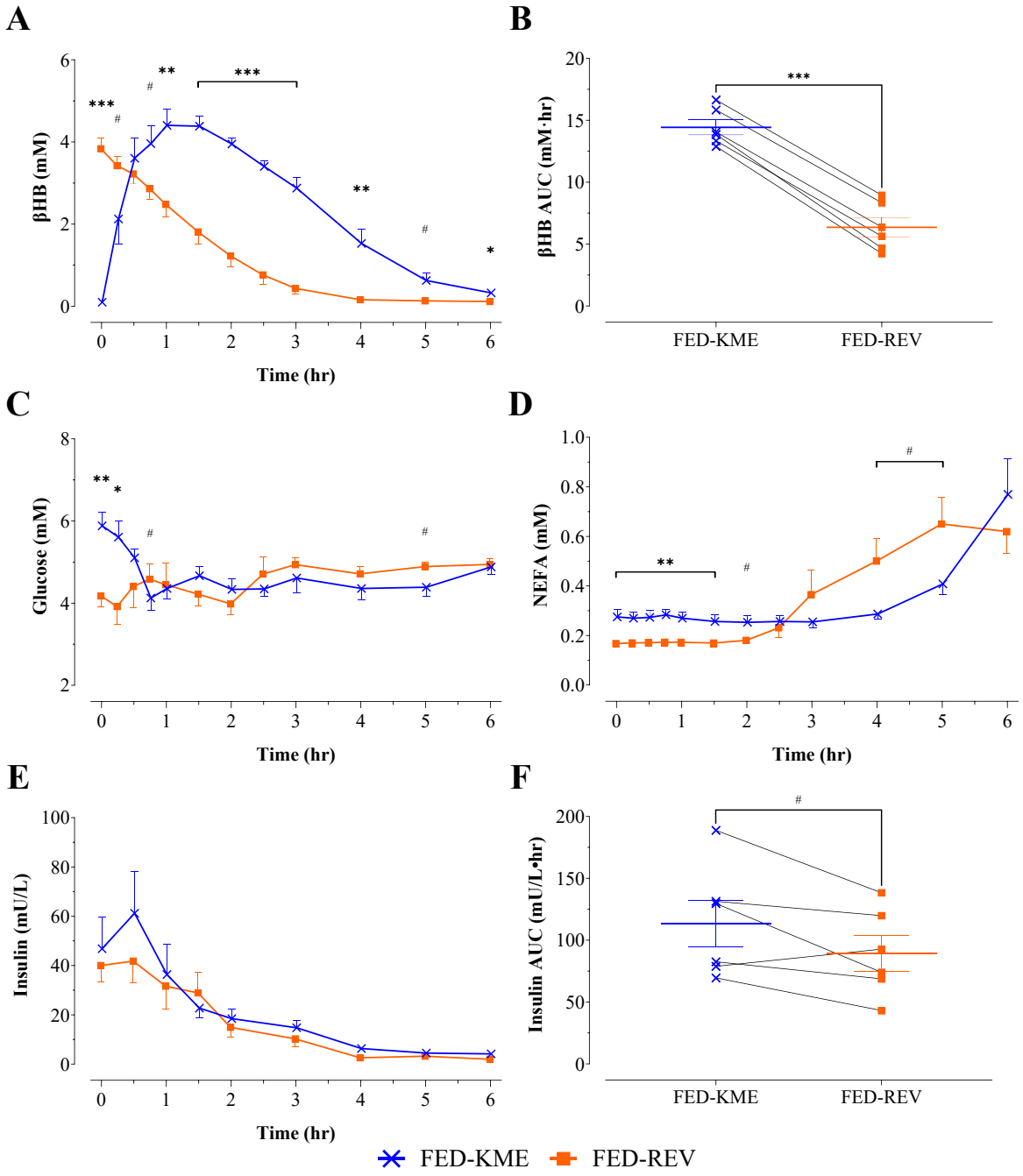


***Figure 5.11* - Plasma βHB, glucose, NEFA & insulin during the FED-KME & FED-REV visits.**
*A, βHB concentration (mM); **B**, βHB AUC (mM·hr); **C**, glucose concentration (mM); **D**, NEFA concentration (mM); **E**, insulin concentration (mU/L); **F**, insulin AUC (mU/L·hr). AUC, area-under-the-curve. Data presented as Mean ± SEM (with individual values plotted for B/F). # $p < 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ between FED-KME and FED-REV conditions. n = 6.*

Plasma HDL-cholesterol, non-HDL-cholesterol, and total cholesterol did not exhibit time, condition, or interaction effects ($p \geq 0.133$; Appendix O; non-HDL-cholesterol data not presented), with AUCs for these measures not differing between conditions ($p \geq 0.130$).

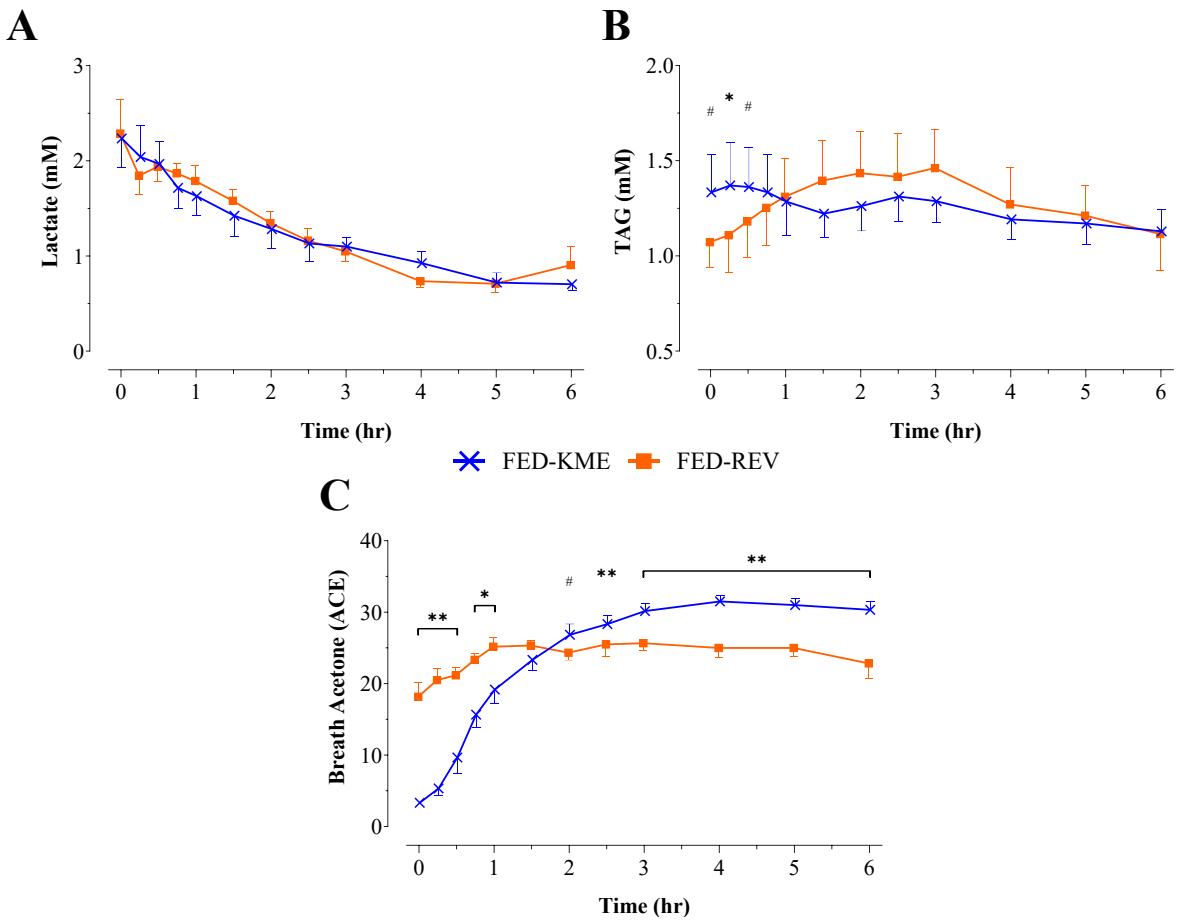


***Figure 5.12* - Plasma lactate, plasma TAG, & breath acetone concentrations during the FED-KME & FED-REV visits.**

A, plasma lactate (mM); ***B****, plasma TAG (mM);* ***D****, breath acetone (ACE; arbitrary units). Data presented as Mean ± SEM.* $^{\#}$ *p < 0.05,* ***** *p < 0.05,* ****** *p < 0.01 between FED-KME and FED-REV conditions. n = 6.*

ΔβHB-AUC (Δ represents FED-KME-AUC *minus* FED-REV-AUC for a given metric) did not predict ΔAUCs for any circulating metabolite ($p \geq 0.263$). Δlactate-AUC and ΔHDL-cholesterol-AUC were found to negatively predict each other ($R^2 = 0.863$; $p = 0.007$).

Urine Biochemistry

Total urine volume and total βHB excreted mass were not different between conditions ($p \geq 0.148$; *Figure 5.13*).

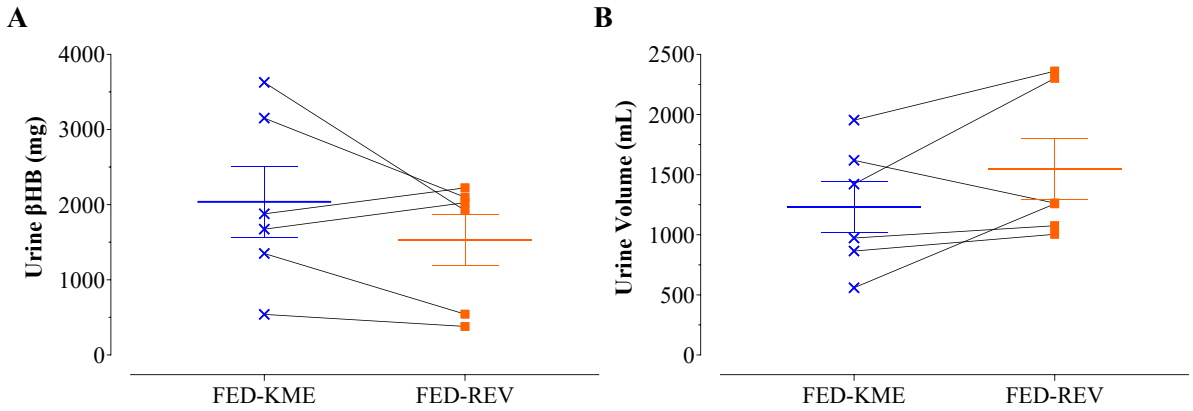


***Figure 5.13* - Urine βHB excretion & volume across the FED-KME & FED-REV visits.**
***A**, urine βHB (mg); **B**, urine volume (mL). Urine collected from: FED-KME, 0 min to 6 hr; FED-REV, -2 hr to 4 hr. Data presented as Mean ± SEM with individual values plotted. n = 6.*

Respiratory, Appetite, & Gastrointestinal Distress Measures

Effects of time but not of condition, nor interaction effects, were observed for $\dot{V}O_2$, $\dot{V}CO_2$, RER, BF, V_T, and V_E (Appendix O).

All appetite metric scores were similar between conditions when overnight fasted for each visit ($p \geq 0.420$). Time effects were established for all appetite measures ($p < 0.001$). *Hunger* presented condition and interaction effects ($p \leq 0.035$; *Figure 5.14-A*), whilst *Total Appetite* carried trends for both effects ($p \leq 0.065$; *Figure 5.14-E*) with post-hoc analyses conducted as this was exploratory work. A weak trend for an effect of condition was observed for *Fullness* ($p = 0.072$; *Figure 5.14-B*) in the absence of an interaction effect ($p = 0.814$). *Satisfied* carried an interaction effect ($p = 0.011$; *Figure 5.14-C*) without a condition effect ($p = 0.276$). Both condition and interaction effects were absent for *How Much Could Eat* ($p \geq 0.100$; *Figure 5.14-D*).

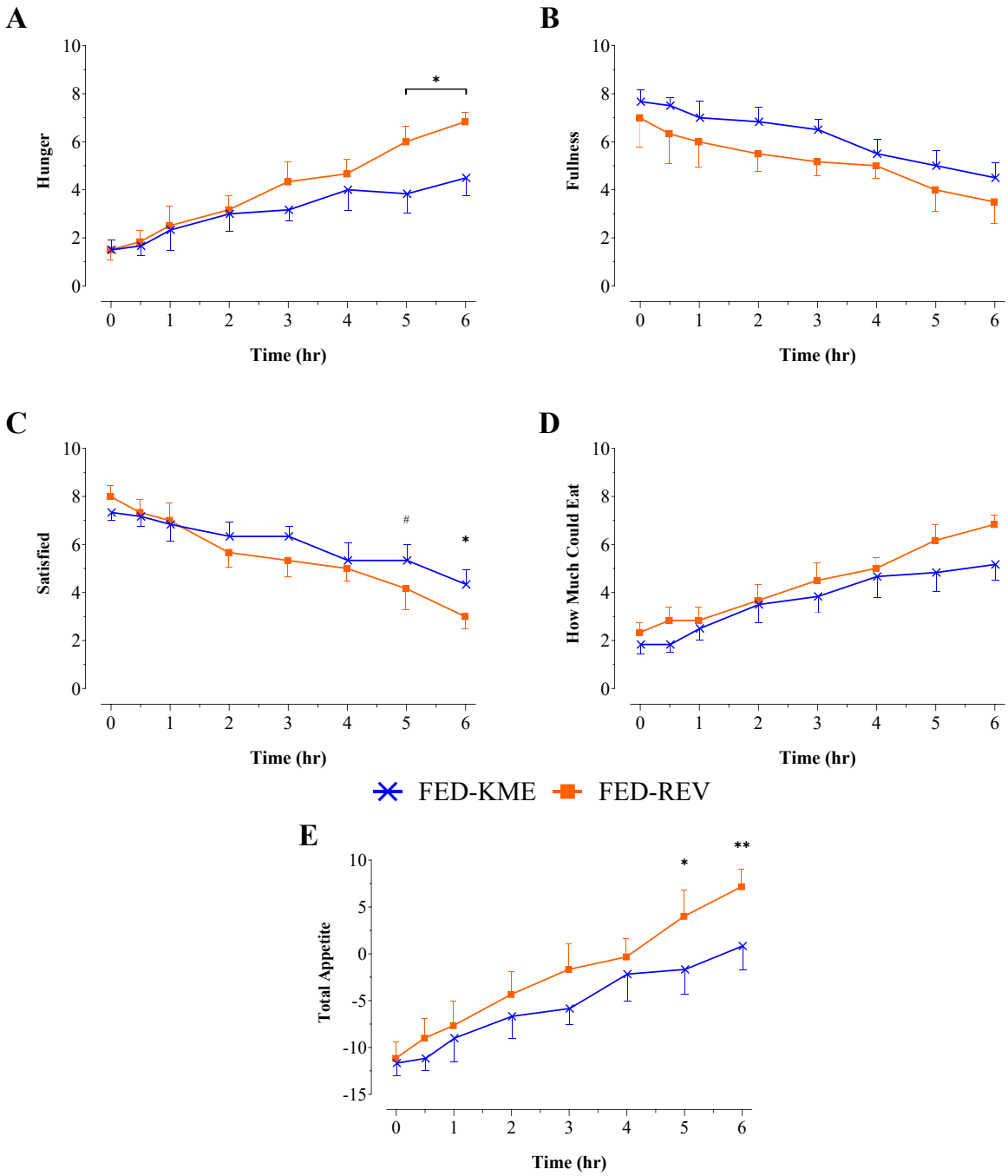


Figure 5.14 *- Appetite metrics across the FED-KME & FED-REV visits.*
A, *Hunger;* ***B,*** *Fullness;* ***C,*** *Satisfied;* ***D,*** *How Much Could Eat;* ***E,*** *Total appetite, composite score of the four appetite metrics (exploratory post-hoc analyses). Data presented as Mean ± SEM; 0-10 scale (A-D).* $^{\#}$ *$p < 0.05$,* * *$p < 0.05$,* ** *$p < 0.01$ between FED-KME and FED-PLA conditions. $n = 6$.*

No time, condition, or interaction effects were established for GI distress ($p \geq 0.722$) with total symptom scores not elevated above 0.3 at any timepoint for either condition.

5.3.3 FED-KME vs FAST-KME Visits (βHB Profiles)

These visits were compared to establish how feeding state might influence plasma βHB profiles after ingestion of the same 573 mg/kg·BW dose of KME.

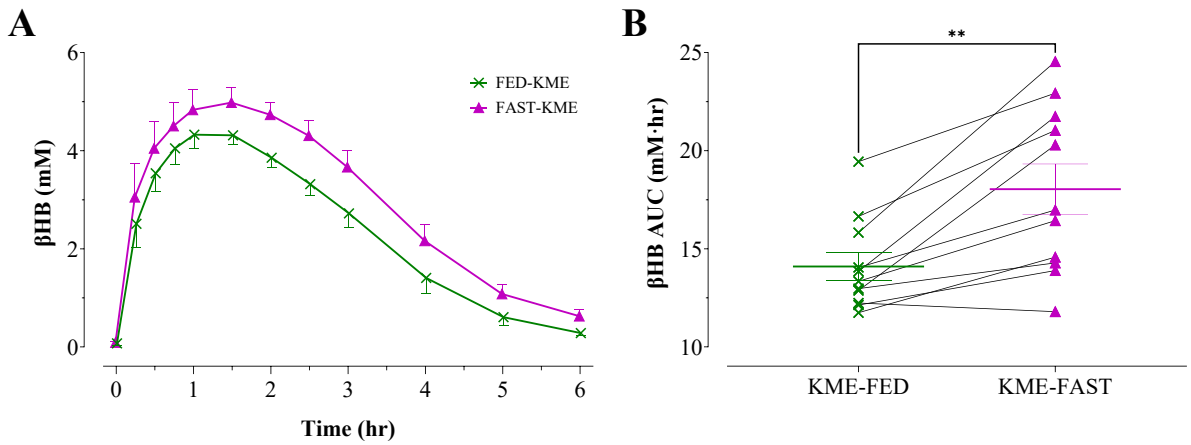


***Figure 5.15* - Plasma βHB during the FED-KME & FAST-KME visits.**
***A**, βHB concentration (mM); **B**, βHB AUC (mM·hr). AUC, area-under-the-curve. Data presented as Mean ± SEM (with individual values plotted for B). * p < 0.05, ** p < 0.01, *** p < 0.001 between FED-KME and FAST-KME conditions. n = 11.*

Plasma βHB exhibited effects of time ($p < 0.001$; *Figure 5.15-A*) and condition ($p = 0.013$), with concentration and βHB-AUC ($p = 0.001$; *Figure 5.15-B*) greater during FAST-KME compared to FED-KME, though no interaction effect was present ($p = 0.446$). Whilst the time to peak concentration (T_{max}) did not differ between conditions (FED-KME: 75.00 ± 9.83 min; FAST-KME: 79.09 ± 12.95 min; $p = 0.818$), the maximal concentration attained (C_{max}) was greater for FAST-KME (FED-KME: 4.678 ± 0.209 mM; FAST-KME: 5.571 ± 0.335 mM; $p = 0.047$).

5.3.4 FAST-KME vs FAST-PLA Visits

Fasted visits were compared to assess the impact of exogenous ketosis on wider circulating metabolism in isolation from ingestion of other macronutrients.

Plasma Biochemistry

Plasma βHB concentration exhibited effects of time and condition, alongside a time-x-condition interaction effect ($p < 0.001$; *Figure 5.16-A)*, with βHB-AUC greater under FAST-KME compared to FAST-PLA ($p < 0.001$; *Figure 5.16-B*). ΔβHB-AUC (Δ represents FAST-KME-AUC *minus* FAST-PLA-AUC for a given metric) was not predicted by age, height, weight, or BMI ($p \geq 0.172$). At an assumed 0.54 g/hr rate of βHB oxidation[71], 78.75 ± 2.92% of βHB disposal was unexplained across 0-6 hr during FAST-KME, equating to 40.88 ± 4.22 g. If AcAc in circulation was assumed at 0.6 mM this explained only a further 0.16 ± 0.04% of βHB disposal. Alternatively, AcAc at 2.0 mM explained 2.12 ± 0.07%. Circulating glucose demonstrated time, condition, and interaction effects ($p < 0.001$; *Figure 5.16-C*), with concentration and glucose-AUC ($p < 0.001$; *Figure 5.16-D*) lower for FAST-KME compared to FAST-PLA. Time, condition, and interaction effects were also present for plasma insulin ($p \leq 0.002$; *Figure 5.16-E*) with concentration, thus insulin-AUC ($p = 0.003$; *Figure 5.16-F*), greater under FAST-KME.

Plasma NEFA exhibited time, condition, and interaction effects ($p < 0.001$; *Figure 5.17-A*), with NEFA-AUC lower under FAST-KME ($p < 0.001$; *Figure 5.17-B*). Correspondingly, time, condition, and interaction effects were observed for circulating glycerol ($p < 0.001$; Appendix P), with glycerol-AUC lower for FAST-KME ($p < 0.001$; Appendix P). Plasma lactate demonstrated time and condition ($p \leq 0.041$; *Figure 5.17-E*), but not interaction ($p = 0.142$), effects, with concentration and lactate-AUC ($p = 0.005$; *Figure 5.17-F*) found to be significantly greater under FAST-KME compared to FAST-PLA.

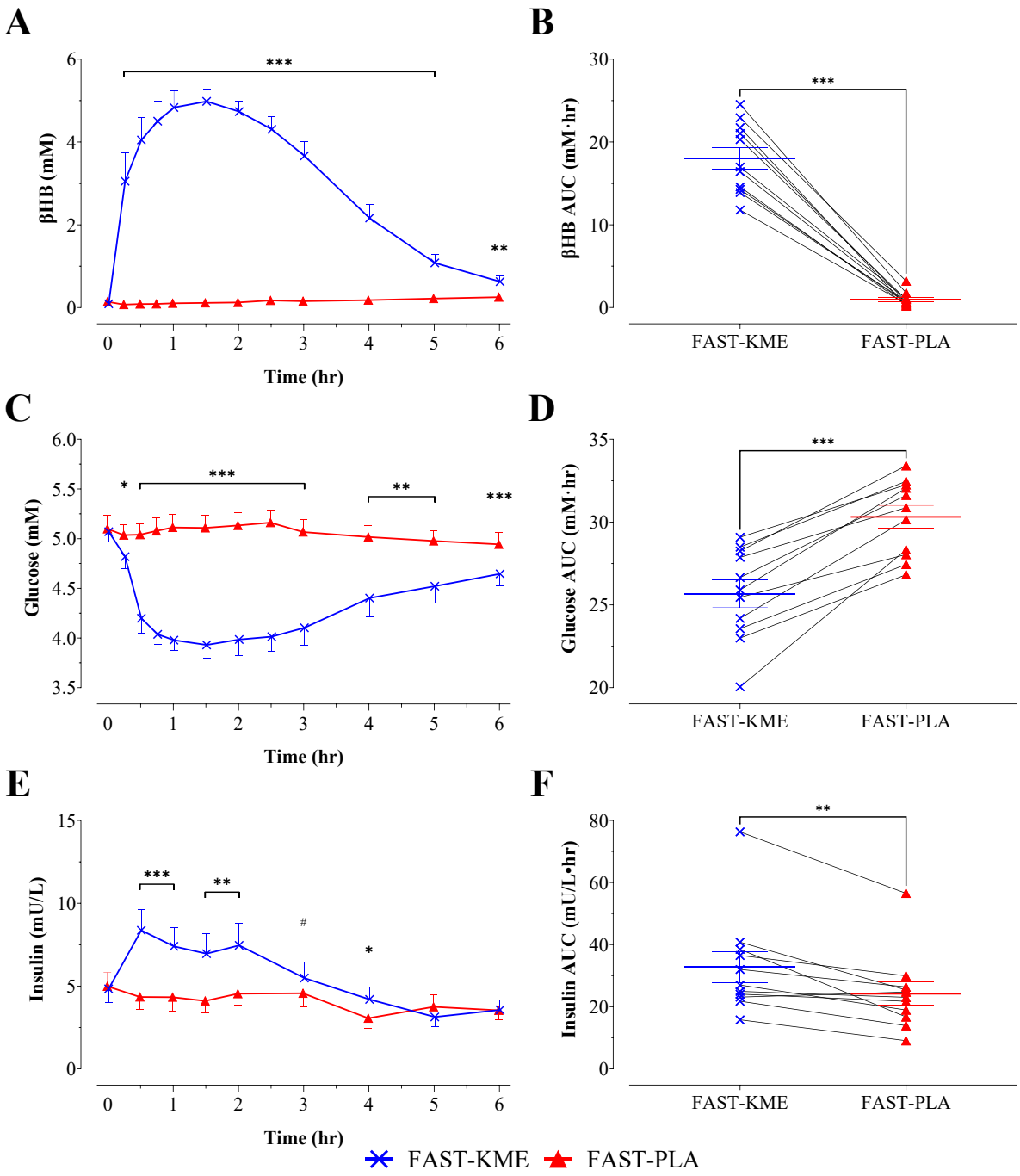


***Figure 5.16* - Plasma βHB, glucose, & insulin during the FAST-KME & FAST-PLA visits.**

*__A__, βHB concentration (mM); __B__, βHB AUC (mM·hr); __C__, glucose concentration (mM); __D__, glucose AUC (mM·hr); __E__, insulin concentration (mU/L); __F__, insulin AUC (mU/L·hr). AUC, area-under-the-curve. Data presented as Mean ± SEM (with individual values plotted for B/D/F). # $p < 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ between FAST-KME and FAST-PLA conditions. n = 11.*

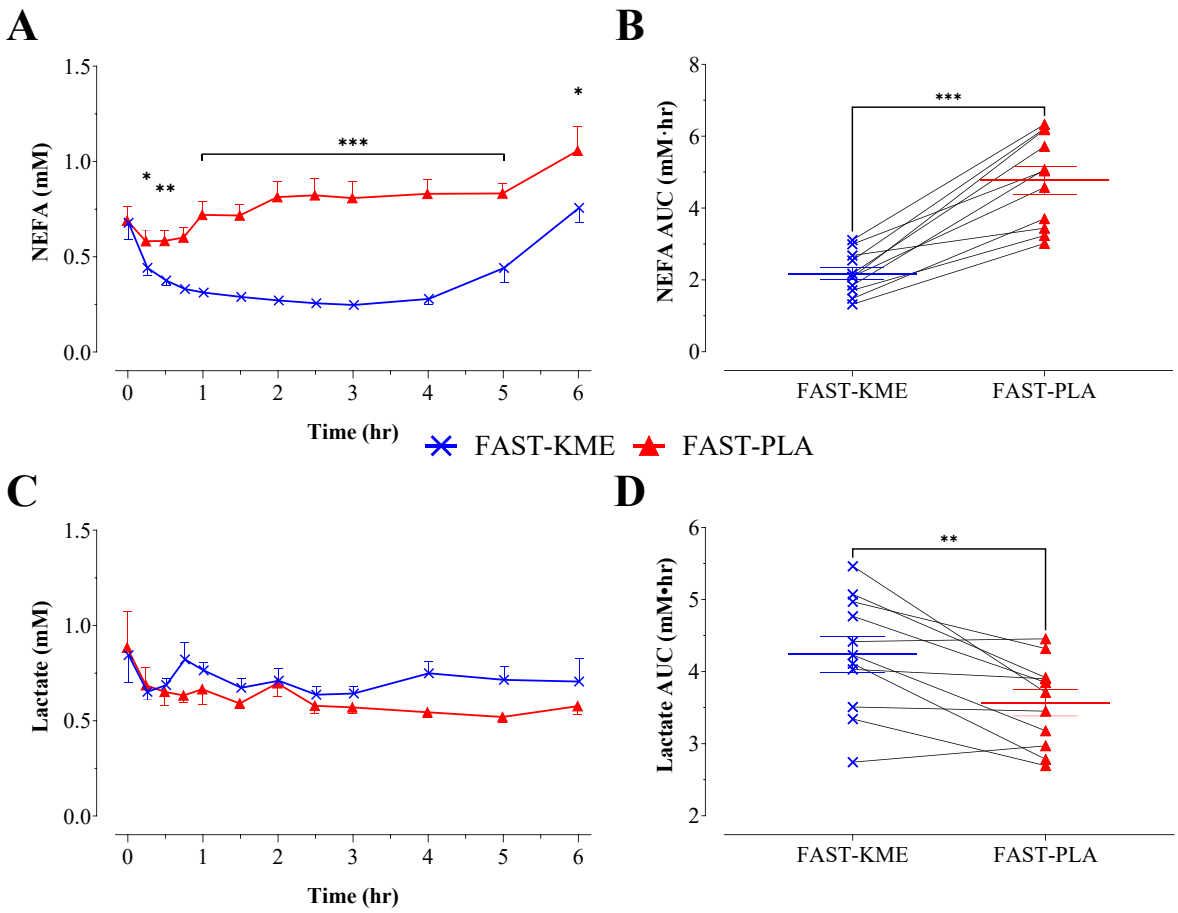


***Figure 5.17* - Plasma NEFA & lactate during the FAST-KME & FAST-PLA visits.**

*A, NEFA concentration (mM); **B**, NEFA AUC (mM·hr); **C**, lactate concentration (mM); **D**, lactate AUC (mM·hr). AUC, area-under-the-curve. Data presented as Mean ± SEM (with individual values plotted for B/D). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ between FAST-KME and FAST-PLA conditions. n = 11.*

Plasma urea concentration displayed an effect of time ($p < 0.001$; *Figure 5.18-A*) but not condition or interaction effects ($p \geq 0.123$), and urea-AUC did not differ between conditions ($p = 0.223$). Circulating TAG exhibited time and interaction effects ($p \leq 0.041$; *Figure 5.18-B*), in the absence of an effect of condition ($p = 0.354$), with TAG-AUC not differing between FAST-KME and FAST-PLA ($p = 0.378$). Neither plasma HDL-cholesterol nor total cholesterol exhibited time effects ($p \geq 0.511$; *Figure 5.18-C/E*) though interactions effects were established for both ($p \leq 0.020$). A condition effect was present for HDL-cholesterol ($p = 0.049$), alongside a trend for a condition effect in total cholesterol ($p = 0.060$). Accordingly, HDL-cholesterol-AUC was greater ($p = 0.035$), and there was a trend for total cholesterol-AUC being greater ($p = 0.067$), under FAST-KME.

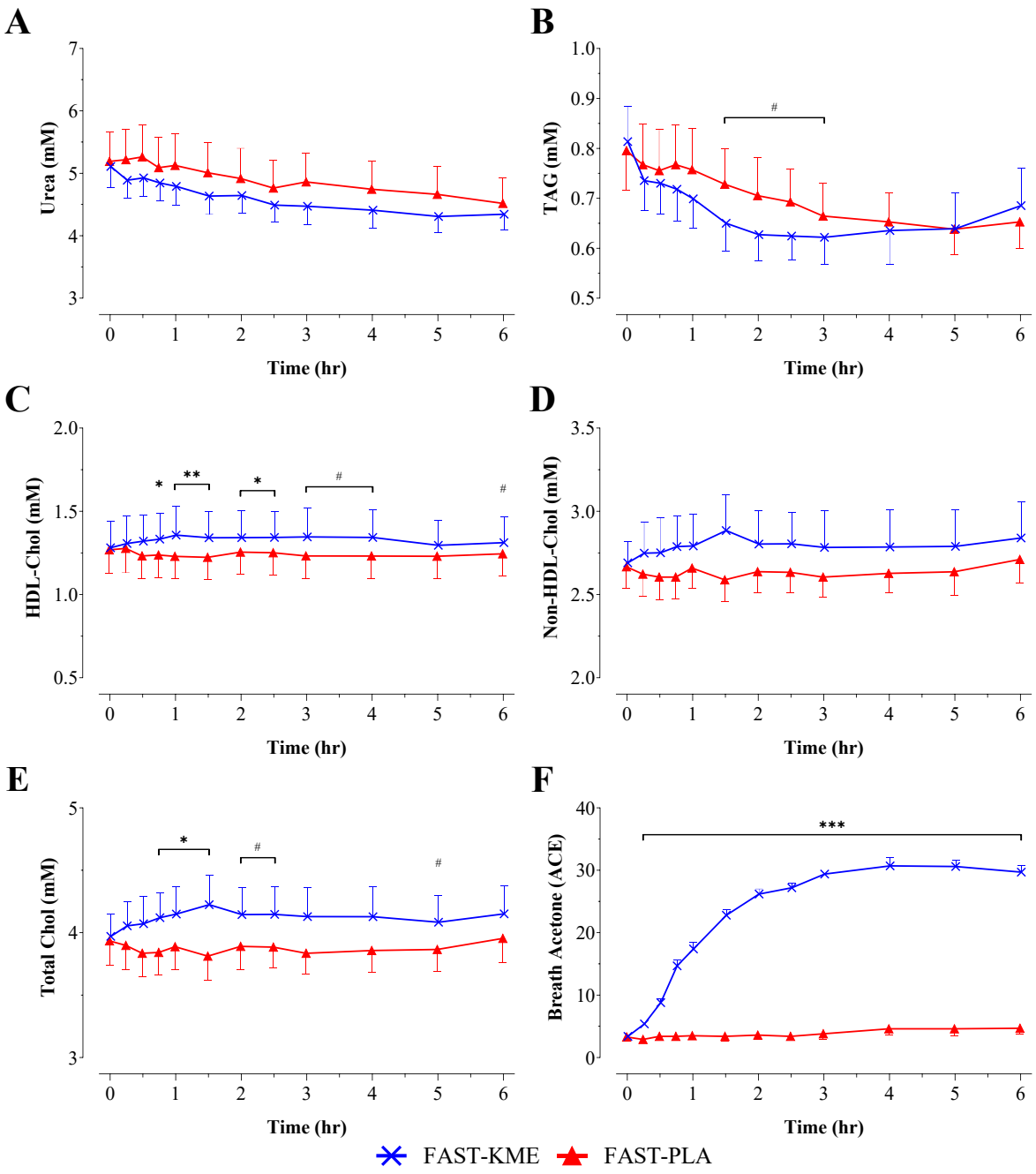


***Figure 5.18* - Plasma metabolite & breath acetone concentrations during the FAST-KME & FAST-PLA visits.**

*A, plasma urea (mM); B, plasma TAG (mM); C, plasma HDL-chol (mM); D, plasma non-HDL-chol (mM); E, plasma total chol (mM); F, breath acetone (ACE; arbitrary units). Chol, cholesterol. Data presented as Mean ± SEM. # $p < 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ between FAST-KME and FAST-PLA conditions. n = 11.*

No time, condition, or interaction effects were observed for plasma non-HDL-cholesterol ($p \geq 0.191$; *Figure 5.18-D*), with non-HDL-cholesterol-AUC not differing between conditions ($p = 0.204$). Breath

acetone presented time, condition, and interaction effects ($p < 0.001$; *Figure 5.18-F*), with an acetone-AUC condition effect revealing greater concentrations under FAST-KME ($p < 0.001$).

ΔβHB-AUC did not predict ΔAUCs for any circulating metabolite ($p \geq 0.123$). ΔGlucose-AUC and ΔTAG-AUC (*Figure 5.19-A*), ΔNEFA-AUC and Δglycerol-AUC ($R^2 = 0.878$; $p < 0.001$), along with ΔNEFA-AUC and Δlactate-AUC (*Figure 5.19-B*) positively predicted each other. Δurea-AUC and ΔHDL-cholesterol-AUC were found be negatively associated ($R^2 = 0.412$; $p = 0.033$).

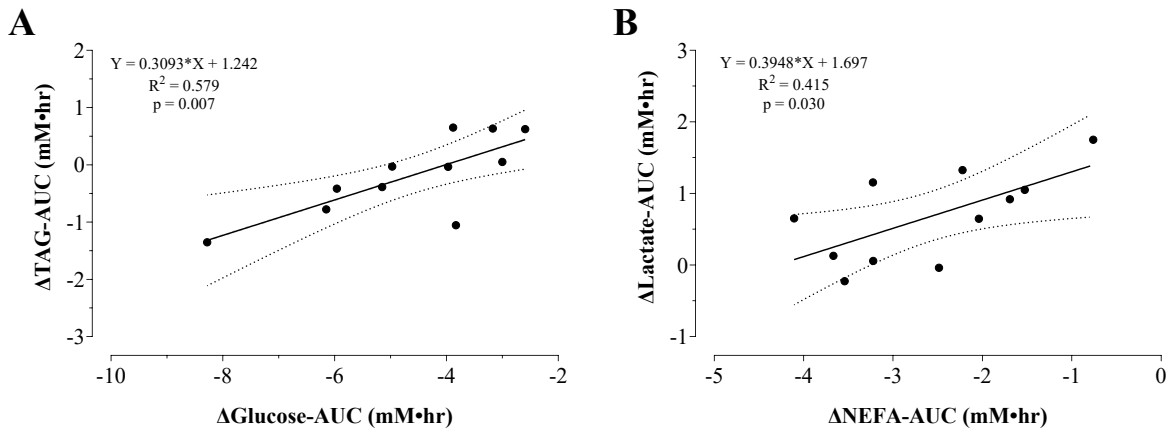


***Figure 5.19* - ΔGlucose-AUC vs ΔTAG-AUC & ΔNEFA-AUC vs ΔLactate-AUC across the FAST-KME & FAST-PLA visits.**

Δ, between condition difference calculated as FAST-KME minus FAST-PLA; AUC, area-under-the-curve. Solid line, linear best fit; dashed lines, 95% confidence intervals. n = 11.

Urine Biochemistry

Total βHB mass excreted within urine was greater across the FAST-KME visits compared to FAST-PLA ($p < 0.001$; *Figure 5.20-A*), whilst total visit urine volume was lower for FAST-KME ($p = 0.017$; *Figure 5.20-C*). Excreted urea mass was not different between conditions ($p = 0.605$; *Figure 5.20-B*).

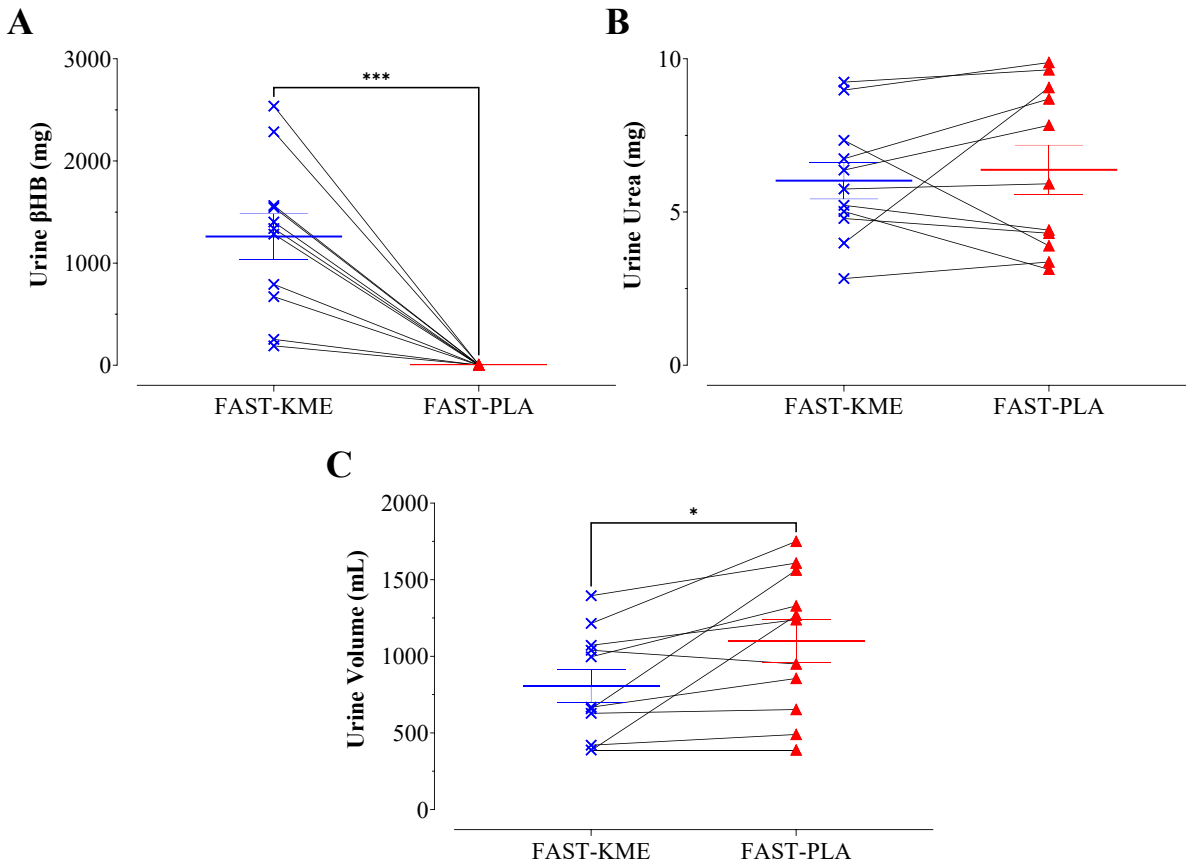


Figure 5.20* - *Urine metabolite excretion & volume across the FAST-KME & FAST-PLA visits.** *A, urine βHB (mg); **B**, urine urea (mg); **C**, urine volume (mL). Urine collected from 0 min to 6 hr. Data presented as Mean ± SEM with individual values plotted. * $p < 0.05$, *** $p < 0.001$ between FAST-KME and FAST-PLA conditions. n = 11.*

Respiratory, Appetite, & GI Distress Measures

Time effects were absent for $\dot{V}O_2$, BF, and V_T, but present for $\dot{V}CO_2$ and V_E, with a weak trend for an effect for RER, whilst no condition or interaction effects were observed for any respiratory measure (Appendix P).

Time effects were established for all appetite metrics ($p < 0.001$; Appendix P), though no condition or interaction effects were observed ($p \geq 0.412$).

No time, condition, or interaction effects were established for GI distress ($p \geq 0.911$), with total symptom scores not elevated above 0.3 at any timepoint for either condition.

5.3.5 Blinding Efficacy

FED-KME & FED-PLA Visits

Of the eleven participants, four correctly identified visit condition order, reporting an average certainty of 3.7. Those who guessed incorrectly provided an average certainty rating of 4.0. Therefore, condition blinding appeared to be effective for these visits.

FAST-KME & FAST-PLA Visits

Five of the eleven participants correctly identified visit condition order, with an average certainty of 3.0. Those who guessed incorrectly presented an average certainty of 2.2. Thus, condition blinding appeared to be efficacious for these visits also.

5.4 Discussion

This study investigated how exogenous ketosis might influence resting hepatic and systemic metabolism in healthy adults. It was discovered that, after a two day high-carbohydrate high-sugar diet, consuming the KME lead to prolonged excursion in elevation of appearance of newly synthesised palmitate in VLDL-TAG compared to placebo, indicating increased hepatic DNL. Raised circulating insulin levels, alongside supressed glucose and NEFA, were also seen during ketosis in both fed and fasted conditions, whilst the KME elevated HDL-cholesterol in the fasted state only. When contrasting ketotic profiles in fed and fasted conditions, no difference was found for plasma βHB concentration T_{max}, but C_{max} and βHB-AUC were lower postprandially.

5.4.1 Postprandial Lipid Metabolism

This work was the first to explore hepatic lipid metabolism under exogenous ketosis in humans, finding that postprandial hepatic DNL was elevated by 49.0% at 3 hr, and 76.4% at 6 hr (relative % increase), after the KME had been ingested compared to PLA.

These findings were observed within the context of the two day high-carbohydrate high-sugar isocaloric diet that the participants consumed before each visit to both upregulate and standardise basal DNL. Prior to this investigation, it was unclear whether DNL might be acutely increased or lowered by exogenous ketosis. Plasma βHB was positively associated with proportional saturation of circulating FAs in *Chapter 3*, indicating that DNL may have been upregulated by the KME. Conversely, ketosis was observed to lower circulating glucose at rest and during exercise in the absence of changes to circulating insulin[318] in *Chapter 3*, and during exercise in *Chapter 4*, in alignment with past studies[37]. This would reduce availability of glucose as a primary lipogenic substrate[306] and potentially supress DNL. Therefore, this high-carbohydrate high-sugar diet was chosen to upregulate lipogenic pathways[301,304,582,608,609], in young and disease-free participants for whom DNL would otherwise be expected to be low[302]. This provided scope for lipogenesis to potentially be lowered by ketosis, with this setting of upregulated basal lipogenesis additionally reflecting the chronically elevated DNL seen in many metabolic disorders[302,331,573] without the confounder of disease state in the population studied.

Evidencing this diet-induced upregulation, fasted DNL was ~twofold greater than what might be expected in the same population consuming a habitual diet[302].

Observations in the current study of increased postprandial hepatic DNL under exogenous ketosis are potentially explained by the elevated circulating insulin stimulating the pyruvate-fed mitochondrial to cytosolic citrate lipogenic pathway[57]. Greater insulin levels in FED-KME, compared to FED-PLA, could have upregulated DNL by driving increased hepatic glycolysis, and therefore availability of cytosolic citrate (thus Ac-CoA) as a lipogenic substrate. Additionally, elevated insulin signalling, in combination with low plasma NEFA availability, may have activated the transcription factor SREBP-1c, augmenting expression of key lipogenic enzymes ACC and FASN[309,430]. Exploratory insulin kinetics analysis in two individuals, presented in this chapter, indicated that ketosis might reduce ICR compared to PLA, which may be a factor in the observed hyperinsulinemia, however it did not appear to explain the KME's influence on DNL. Observations of ICR being lower under ketosis sits in contrast to prior findings of elevated insulin in the hepatic portal vein, but not systemically, under ketosis[610], indicating commensurately increased insulin secretion and clearance. Whilst it was not assessed here, exogenous ketosis has been observed to elevate glucagon secretion, even in the absence of changes to circulating insulin[158,194,611,612], with a weak trend seen for elevated glucagon under High-KME in *Chapter 3* without any between-condition differences in insulin. As greater glucagon would inhibit malonyl-CoA formation[613], and therefore act to supress DNL, kinetics of insulin and glucagon, and their relative contributions to glucose and lipid metabolism during ketosis, warrant further investigation.

Although plasma ΔβHB-AUC positively predicted Δinsulin-AUC, neither correlated with ΔDNL-AUC, suggesting the lipogenic response observed in my work was multifactorial. The 1,3-butanediol component of the KME, metabolised similarly to ethanol[196,215], could have also stimulated DNL. Its oxidation to βHB and AcAc via alcohol dehydrogenase and aldehyde dehydrogenase generates reducing equivalents by increasing the NADH:NAD^+ ratio, in turn potentially blunting SIRT-1 and AMPK activity, thus disinhibiting acetyl-CoA carboxylases (ACACA)[123,196,215]. However, 1,3-butanediol oxidation has been previously shown to supress hepatic DNL in rats[614], so its potential influence here is not clear. Additionally, suppression of ketogenesis under exogenous ketosis[105] may have left greater citrate available for lipogenesis, potentially contributing to elevated DNL. In support of this, reductions

in expression of the ketogenic enzyme HMGCS2 have been shown to increase DNL in mature mice fed a high-fat diet[615]. Furthermore, glutamine, the most abundant circulating amino acid[298,417,418] which was elevated in a plasma βHB dose-dependent manner in *Chapter 3*, can act as a lipogenic carbon source *in vitro*[616], though *in vivo* evidence is lacking.

Though ketone oxidation is unlikely to have influenced DNL through the mitochondrial to cytosolic citrate pathway as hepatic ketolytic capacity is low[38], AcAc can act directly as a lipogenic substrate through the acetoacetyl-CoA synthetase (AACS) pathway[48,57]. Though plasma AcAc was not assessed here, KBs were likely circulating 4:1 βHB:AcAc during FED-KME[23], therefore hepatocyte cytosolic AcAc levels would be expected to be equally elevated[218], with AcAc able to enter the lipogenic pathway through conversion to AcAc-CoA by AACS, then to Ac-CoA by acetyl-CoA acetyl-transferase 2 (ACAT2)[29,428]. Though AACS is saturated by AcAc at relatively low concentrations (K_M ~50 μM[617]), it is very highly expressed and active in the cytoplasm of hepatocytes, having been measured in humans at 37.6 μM[618], therefore elevated flux through this pathway is possible consequent of increased AcAc availability during exogenous ketosis. In support of this, work in perfused rat livers has shown that KBs contribute up to 22% of the carbon required for the synthesis of FAs[619], and to 15% of FA synthesis in *ex vivo* isolated hepatic parenchymal cells collected from starved rats[56]. Rauckhorst et al.[57] have suggested that the AcAc-fed and citrate-fed pathways might act reciprocally, with lipogenesis from ketone precursors upregulated when mitochondrial citrate transport is disrupted. Therefore, if citrate-fed lipogenesis was blunted by supressed availability of circulating glucose and NEFA for hepatic uptake and oxidation, even with increased circulating insulin, this might disinhibit the AACS pathway and elevate AcAc-fed lipogenesis. ^{13}C tracer methodologies could potentially be utilised to explore this hypothesis[57].

No between-condition differences were observed in plasma VLDL-TAG or TAG concentrations, suggesting that net VLDL secretion rates may not have been affected by ketosis. Whilst elevated insulin under FED-KME might have been expected to supress VLDL secretion[620], it was not possible to quantify secretion and clearance rates here. This could be assessed in future work by employing infusions of labelled glycerol, palmitate, and leucine[621], especially in the context of ketosis supressing circulating NEFA availability for hepatic uptake and therefore potential incorporation into VLDL-TAG[297].

5.4.2 Plasma βHB Profiles

Plasma βHB concentrations were compared between the FED-KME and FAST-KME visits to explore how feeding state might impact metabolism of the KME. Though no difference was seen between nutritional states for T_{max}, C_{max} was ~0.9 mM lower, and βHB-AUC was reduced by 21.9%, when the KME was ingested in a postprandial state compared to when fasted. This indicates that, if specific circulating KB levels are being targeted, for example for sporting performance[24], then KME dosing may need to be adjusted based on adjacency to prior feeding. Plausible mechanisms that might underpin these observations include delayed gastric emptying/gut transit[207], competition for gut esterases and gut luminal MCTs[208,213], and slowed hepatic oxidation of 1,3-butanediol[110,121–124,215] when fed compared to fasted.

My findings are in agreement with work by Stubbs et al.[23], who provisioned the KME at 395 mg/kg·BW, ingested immediately following a 1.07 g/kg·BW carbohydrate breakfast meal. Despite substantial methodological differences compared to work presented in this chapter, Stubbs et al.[23] similarly found plasma βHB T_{max} to be unaffected by feeding state and C_{max} to be ~1 mM lower postprandially compared to fasted. However, observation, in my work, of plasma βHB rising to 4.1 mM at 45 min under FED-KME sits in contrast to the High-KME condition in *Chapter 3* where βHB had only risen to 2.4 mM after 50 min, despite the protocols sharing exactly the same relative timings, KME dose, and breakfast composition. The only difference was that the KME drink in *Chapter 3* was consumed alongside 25 g of carbohydrate. As this was provisioned as an 8.3% solution[622], gastric emptying, and therefore βHB appearance in the bloodstream, may have been slowed, further evidencing the impact that carbohydrate ingestion might have on metabolism of the KME, and potentially vice versa.

The mechanisms and kinetics concerning non-oxidative βHB metabolism under exogenous ketosis remain poorly understood. Here 79% of βHB disposal was unaccounted for in FAST-KME, even at an assumed βHB oxidation rate of 0.54 g/hr[71], with negligible excretion in urine. Breath acetone findings provide some insight, with concentrations substantially elevated for KME conditions under both FED and FASTED nutritional states, only peaking at 3-4 hr and not tangibly declining at 6 hr. This indicates that the release of acetone, formed through spontaneous decarboxylation of AcAc[286], may represent a substantial pathway of ketone disposal, with its appearance in breath lagging behind changes to plasma

βHB likely due to it being sequestered into adipose tissue[23,286]. Breath acetone-AUC was the same between FED-KME and FAST-KME, however, so does not appear to help explain why βHB-AUC differed between feeding states. Additionally, breath acetone plateaued under FED-REV at a 15-20% lower level than in FED-KME, suggesting that systemic AcAc levels may have been lower in FED-REV. Future work could measure acetone release across a much longer timeframe, as well as utilising ^{13}C-KME to trace βHB and AcAc metabolism, which as yet has only been employed in an exercise setting[70,71], alongside measuring circulating AcAc concentration[23,214] to provide a greater breadth of insight into KB disposal.

5.4.3 Fasted Lipid Metabolism

In a fasted state, but not postprandially, HDL-cholesterol and total cholesterol levels were increased by exogenous ketosis, alongside indications that plasma TAG may have been lowered, with non-HDL-cholesterol unaffected. The only other study to assess the impact of exogenous ketosis on these circulating lipid metrics observed a lowering of circulating TAG, but no change in total cholesterol nor in HDL-cholesterol, at ~3.5 mM βHB compared to a non-caloric placebo[570]. However, participants there were pre-diabetic so findings may not be directly comparable to work presented here.

In the current study, strong trends indicated that plasma TAG levels might have been lowered from 90-150 min during exogenous ketosis. This may have been resultant of elevated insulin supressing VLDL secretion[620] and/or increasing adipose LPL activity[623,624], therefore TAG clearance from VLDL into adipose tissue[625]. Additionally, suppressed plasma NEFA and glycerol levels under FED-KME may have diminished hepatic uptake of FAs for re-esterification[626], reducing IHTAG availability for secretion within VLDL-TAG[430].

Circulating HDL-cholesterol being elevated after KME ingestion compared to placebo in my work sits in agreement with Caminhotto et al.[627] who discovered that HDL-cholesterol levels were increased after both acute and chronic βHB supplementation in rats. As the authors[627] established that βHB was able to activate GPR10A receptors, it is likely that βHB acts similarly to nicotinic acid[105], prolonging the half-life of HDL by inhibiting hepatic lipoprotein uptake[628]. Additionally, if VLDL-TAG secretion was

lowered under FAST-KME, this might act to reduce the transfer of TAG from VLDL to HDL, lowering rates of HDL hydrolysis by hepatic lipases[629]. Furthermore, elevated plasma insulin, alongside the lowered circulating NEFA levels, may have inhibited cholesteryl ester transfer protein (CETP)-facilitated transfer of cholesterol-esters from HDL to VLDL and LDL particles[630]. Though it has not yet been investigated *in vivo*, βHB may also be able to induce adiponectin secretion through activation of GPR10A receptors[631], which could elevate HDL-cholesterol levels by facilitating both HDL biogenesis and increased cholesterol efflux from peripheral tissue into HDL[632].

Though plasma TAG, HDL-cholesterol, and total cholesterol concentrations remained within normal ranges under both FAST-KME and FAST-PLA conditions, the population studied were metabolically healthy. If plasma HDL-cholesterol was similarly elevated by ~0.1 mM by the KME in patients with T2D, and if the elevation was maintained under prolonged exogenous ketosis, this increase in HDL-cholesterol has been associated with a 15% reduction in coronary artery disease risk[633], whilst, in combination with potentially lowered TAG, may slow the onset of insulin resistance[634]. Therefore, further investigation is warranted exploring if the effects of acute KME dosing might be maintained chronically, especially in those with dyslipidaemia. Future work could also endeavour to ascertain if LDL-cholesterol and remnant (IDL/VLDL) cholesterol are impacted by exogenous ketosis[570,635,636], as rodent studies indicate that elevated hepatic AcAc could increase *de novo* cholesterogenesis[56,617] and might therefore influence cholesterol-rich particle secretion.

5.4.4 Glucose Metabolism

In work presented here, the lowering in circulating glucose levels seen under exogenous ketosis in both feeding states was likely, at least in part, driven by elevated insulin increasing peripheral glucose uptake, with reductions in plasma NEFA potentially augmenting this effect through increasing insulin sensitivity[637]. Findings in this chapter of KME ingestion elevating plasma insulin in fasted healthy participants align with prior studies[23,162,412], though this effect is not universally observed[194]. As no other macronutrients were consumed alongside the KME during the FAST visits, this indicates that ketosis can stimulate insulin secretion directly, rather than potentiating glucose-stimulated release. This is supported by *ex vivo* studies showing βHB to have the capacity to activate G-protein-coupled receptors

on pancreatic β-cells, elevating intracellular cAMP and Ca^{2+} and leading to insulin exocytosis[72,172–174,638]. Only three studies have explored the effects of KME ingestion on insulin postprandially, however, with one finding it to be elevated[23,416], as was the case in my work, and two seeing no effect[170,416]. As no clear relationship between plasma βHB and insulin concentrations is apparent within this limited data pool, further work is required to characterise the metabolic settings in which ketosis may, or may not, influence insulin kinetics.

Plasma glucose lowering under exogenous ketosis also occurs via insulin independent routes[180], as evidenced by glycaemic levels remaining suppressed in FAST-KME compared to FAST-PLA at 5 hr and 6 hr, when βHB concentrations were still greater under FAST-KME, but insulin levels did not differ between conditions. This might have been consequent of reduced plasma glycerol availability supressing gluconeogenesis[361], whilst alanine, another gluconeogenic substrate, has previously been seen to be lowered under exogenous ketosis[136,639] though circulating amino acid levels were not assessed in this chapter. It is plausible that an aspect of the observed postprandial plasma glucose lowering during exogenous ketosis was consequent of glucose being removed from circulation to provision upregulated lipogenic flux, both in the liver and adipose tissue[640]. Hepatic DNL is unlikely to have produced >10 g of new lipid in this timeframe[641,642], however, so glucose disappearance through conversion to FAs was most probably minor.

Findings from exploratory pilot work within this chapter indicate that an order effect might exist between the KME and ingestion of other macronutrients, with the FED-REV condition's postprandial plasma glucose excursion blunted compared to FED-KME, without insulin levels significantly differing between conditions. This may be indicative of the KME delaying gastric emptying, therefore slowing the appearance of glucose into circulation[305], which would align with findings of lowered GLP-1 and PYY levels seen at rest under ketosis in *Chapter 3*. Whilst rate of gastric emptying has not yet been reported to be influenced by the KME[170,416], it is slowed by direct βHB ingestion[514]. This dynamic could be explored further by utilising ingested paracetamol absorption as a marker of gastric emptying[643–645]. It is also possible that insulin, synergistically potentiated by both the meal and ketosis, had already risen in the hour immediately proceeding the breakfast to a greater extent than in FED-KME and then fallen again by 0 min, increasing glucose uptake in this prior window. Samples were not taken in this hour as

the FED-REV visits were conducted post-hoc, with no comparable data collection timepoints during the FED-KME visits to align with, though findings here may justify further investigation into potential KME-meal order effects.

Plasma lactate was elevated under KME compared to PLA in both FED and FAST conditions in the current study. In the only comparable existing study, lactate was found to be lowered by the KME compared to a calorie free placebo in obese participants undertaking a 2 hr oral glucose tolerance test (OGTT), in the absence of any effect on insulin levels[158]. It may be, therefore, that the elevated insulin seen in my work drove increased glycolysis, hence lactate production[646], as evidenced by lactate and insulin ΔAUCs being strongly positively associated across the postprandial visits. Paradoxically, fasted lactate and NEFA ΔAUCs were also positively related, despite elevated lactate supressing adipose lipolysis by binding to HCAR-1[93] which should have led to a negative relationship. This may therefore represent a type I error, however if it is a valid observation, it potentially represents a novel metabolic relationship under exogenous ketosis.

5.4.5 Strengths & Limitations

One strength of this study was that the data collection periods were of a longer duration than in any comparable work[23,158–160,162,170,416] allowing for more complete assessment of metabolism under exogenous ketosis. Additionally, the postprandial visit KME dose[24,94,107,150,154,229] and breakfast composition[3,5] were highly translatable to endurance exercise settings where exogenous ketosis is currently being both researched and utilised in competition. A further strength was participants having identical diets provided to them for the two days prior to each visit, thus ensuring metabolic standardisation for each visit. This is, however, also a limitation for interpretation of the work, as the high-sugar diet likely upregulated DNL compared to the participants' habitual diets.

Whilst deuterium labelling represents the gold standard approach for *in vivo* assessment of hepatic DNL, the enrichment of non-palmitate *de novo* synthesised FAs such as stearate and oleate was not traced in this current work[609,647]. This may have provided broader insight into the contribution of DNL to the total circulating TAG pool as dynamics of each FA can vary based on nutritional[648] and hormonal[620] state,

and could have been achieved with a longer period of heavy water plasma enrichment[609]. Additionally, the full time-course of postprandial DNL elevation under exogenous ketosis was not captured here, though this was not anticipated when designing the study as elevated DNL excursions are normally observed to return to ~baseline within 3-5 hr[298,317,582,649,650]. Follow-up investigations should therefore look to extend the duration of data collection.

Additionally, the sample size here was too limited to identify metabolic and physiological determinants of βHB kinetic parameters[20,110] in each nutritional state. The influence of sex is unclear, for example, as recent work has shown that female rats metabolise the KME more rapidly than males[121], likely through greater carboxylesterase activity[651], whilst hepatic lipid metabolism is also known to exhibit sex differences[650,652]. Furthermore, inter-individual variability in plasma βHB profiles might have been lessened by providing KME doses based on lean mass, which was not possible to determine here, rather than bodyweight, as the ketolytic capacity of adipose tissue is negligible[38].

5.4.6 Conclusions

In this study, the influence of KME ingestion on circulating and hepatic metabolism was assessed in both fasted and fed states in young, healthy adults at rest. Exogenous ketosis was found to increase the magnitude and duration of postprandial hepatic DNL elevation excursions. As chronically upregulated DNL is linked to worsened outcomes in those with metabolic dysfunction, these findings warrant further investigation in the setting of repeated KME consumption, and in patient populations, to evaluate the impact of increased lipogenesis within the context of observed therapeutic benefits of exogenous ketosis such as improved glycaemic control and reduced circulating NEFA.

Additionally, feeding state was found to impact post-KME ingestion βHB profiles. Findings might inform dose timing and quantity recommendations when ingested subsequent to a high-carbohydrate meal, for example in athletic settings.

Chapter 6 - General Discussion

6.1 Exogenous Ketones for Health and Performance

Diet plays a critical role in determining human health, not only providing substrate for energy production but also modulating signalling pathways and cellular adaptations. One component of health which has come to increased prominence is the physiological capacity, often explored through exercise, with physical fitness a significant predictor of all cause, and cause specific, e.g. cardiovascular disease and cancer, mortality[653]. Accordingly, scientific inquiry into manipulation of diet to best optimise health and performance has expanded in recent years. One nutritional intervention that has been proposed to improve both exercise capacity[7,15,22] and health more broadly[9,654] is the ketogenic diet. Whilst it posited to be the ketosis component that might underpin potential benefits of this diet[38,90,493], exploration into the impact of ketones specifically is confounded by obligate near-elimination of carbohydrate. Instead, exogenous ketone supplements (EKS) present an avenue by which ketosis can be attained without broader dietary manipulation.

This thesis examined how exogenous ketosis, achieved through ingestion of the ketone monoester (KME), might influence human metabolism and physiology both at rest and during endurance exercise, whilst consequently exploring its ergogenic potential for athletic performance. Exercise responses were assessed at differing intensities and KME doses to elucidate settings in which the exogenous ketosis might be recommended to enhance performance, whilst ketotic resting metabolism was characterised in both fasted and postprandial states, reflecting differing scenarios where the KME might be consumed to improve metabolic health.

6.2 Main Thesis Findings

Whilst the KME is garnering increasing interest and adoption within both professional and amateur endurance sport[30,31,228,655], guidelines surrounding its use remain poorly substantiated. *Chapters 3* and *4* therefore investigated how exogenous ketosis might influence exercise capacity, metabolism, and physiology at differing intensities and KME doses.

It was discovered in *Chapter 3* that neither a high nor low dose of the KME impacted postprandial multimodal exercise performance at heavy-to-severe intensities compared to placebo, with athletes also provisioned gold-standard in-exercise carbohydrate. These findings indicated that metabolic and performance benefits of exogenous ketosis at higher exercise intensities, previously seen in a fasted state[70,71,107,150,153], may not translate to a more 'real world' relevant postprandial high-carbohydrate setting. Additionally, circulating glucose levels were seen to be lowered in a plasma βHB concentration-dependent fashion, both at rest and during exercise, even alongside substantive exogenous carbohydrate provision. This glycaemic modulation appears to be insulin-independent and was present even when circulating glucose rose >6 mM during near-exhaustive high intensity exercise.

Again in a postprandial and high-carbohydrate exercise setting, *Chapter 4* utilised prolonged moderate intensity cycling to achieve an ecologically valid state of low glycogen availability, where it is proposed that ketolysis might be better able to materially impact exercise performance[223]. However economy, a key determinant of endurance performance that has been suggested to be improved by ketone oxidation[21,59], was either unaffected or worsened by exogenous ketosis, whilst subsequent high intensity work capacity was not different between KME and placebo conditions. Similar to *Chapter* 3, robust plasma glucose reductions after KME ingestion were observed in *Chapter 4*, with 4 participants even experiencing periods of hypoglycaemia[656]. This again arose despite substantive exogenous glucose provision, and appeared to be independent of ketoacidosis, in agreement with previous work[230].

As the relative contributions of exercise, feeding state, and exogenous ketosis to these findings of lowered circulating glucose were unclear, *Chapter 5* sought to explore how KME ingestion might influence glycaemia at rest in healthy individuals, with and without prior high-carbohydrate feeding. In alignment with prior chapters, plasma glucose levels were suppressed by ketosis compared to a calorie-free placebo, though in contrast to *Chapter 3* circulating insulin was seen to be concomitantly elevated.

This indicated that, without confounders of concurrent carbohydrate ingestion and/or exercise, glucose lowering may be at least in part insulin-driven. *In vivo* glucose metabolism doesn't, however, occur in isolation, so the wider metabolic effects of exogenous ketosis were also explored. Hepatic DNL, for example, represents a key carbohydrate disposal route, and was found to be elevated postprandially during exogenous ketosis, likely driven by increased plasma insulin. Additionally, circulating free fatty acid (FA) levels were substantially lowered in agreement with *Chapter 4*, though not *Chapter 3*, whilst plasma lactate concentrations were elevated at rest, in contrast to being supressed by ketosis during exercise in *Chapter 3*. Findings from *Chapter 5* might therefore act to better inform whether individuals on a high-carbohydrate diet should consume the KME after a meal, as improved glycaemic control may be offset by elevated hepatic DNL and circulating insulin, which are linked to poor metabolic health outcomes[324,567,657], especially in those who might consider consuming the KME therapeutically, such as patients with T2D or pre-diabetes[158,161,329]. Thus, further exploration into the implications of these findings is warranted.

6.3 Future Directions

6.3.1 Buffering Ketoacidosis During Exercise

As findings of worsened cycling economy under exogenous ketosis in *Chapter* 4 may be, in part, due to the ergolytic impact of ketoacidosis[264], a logical next step would be to explore if increasing buffering capacity may lead to alternative performance outcomes. This could be achieved with ingestion of sodium bicarbonate[275]. Though relevant literature is limited, Poffé et al.[157] found KME and bicarbonate co-ingestion improved 15 min TT performance after 3 hr 'intermittent' cycling, whereas the KME alone did not. However, ketosis had fallen to 0.5 mM when the TT was undertaken, so the mechanisms underpinning this finding are unclear and merit further exploration. Additionally, bicarbonate supplementation has not yet been seen to rescue the detrimental impact of ketosis on high intensity exercise performance[152], nor abate ketosis-driven elevations to ventilatory workloads[157,230,234] as seen in *Chapters 3* and *4*. Potential benefits might, though, be unlocked with multi-day bicarbonate supplementation[658], potentially alongside beta-alanine consumption, which can improve intra-cellular buffering capacity[275,659]. Furthermore, calculations to correct $\dot{V}CO_2$ for ketoacidosis-driven blood

bicarbonate depletion were used for the first time in any EKS research in *Chapter 4*. This approach might therefore merit validation, which could be achieved though assessing the kinetics of ^{13}C-bicarbonate elimination[473,660].

6.3.2 Optimal Athlete Population

The athletes studied in *Chapters 3 & 4* were 'Trained/Developmental' or 'Highly Trained/National Level' per the classification framework proposed by McKay et al.[79]. Whilst well-trained endurance athletes are proposed to carry the greatest exercising ketolytic capacity, this is only supported by preliminary human data[71] with prior work conducted in rodents[73,76]. There would therefore be utility in characterising the differential effects of exogenous ketosis in a variety of phenotypes. The impact of sex, for example, remains poorly understood. Whilst *Chapter 4* achieved an ~even sex split, outcomes were underpowered to explore if any inter-sex differences were present, with the majority of previous studies having been male dominated[34] and therefore offer limited insight. It may even be specific patient populations who might benefit most from exogenous ketones with regards to enhancing exercise capacity[661,662], rather than competitive athletes.

6.3.3 Prolonged Exogenous Ketosis for Performance

Whilst exogenous ketones failed to confer beneficial effects to acute exercise performance across *Chapters 3* and *4*, athletes studied were habitually consuming high-carbohydrate diets and therefore likely naïve to ketosis >1 mM[24]. Future work might explore if prolonged ketone exposure, through repeated doses of the KME in the days/weeks prior to being taken for acute exercise, may increase ketolytic enzyme expression/activity and therefore potentiate in-exercise effects of exogenous ketosis. This might increase the magnitude of posited ketone oxidation rate-dependant benefits such that they exceed oxidation-independent detrimental effects (ketoacidosis, increased ventilatory rate, gastrointestinal distress, etc). Beyond this, there is growing interest surrounding exogenous ketosis' application for enhancing post-exercise recovery and potentiating training adaptations[193,457,663]. Suggested benefits include increased glycogen resynthesis rates[168], enhanced muscle anabolism[171], and

blunted symptoms/drivers of non-functional overreaching[193]. Future focus for exogenous ketosis within exercise-related research may, therefore, pivot towards probing modulation of post-, rather than intra-, exercise metabolism by ketosis.

6.3.4 Metabolic Health

Findings of increased postprandial DNL under exogenous ketosis were presented in *Chapter 5*. These were, however, established after a single KME dose and in healthy individuals on a high-sugar diet. The logical next step would therefore be to examine if these findings are replicated in patients with metabolic dysfunction who may consider consuming the KME therapeutically, and who might already have constitutively upregulated DNL, akin to the acute elevation achieved with the free sugar enriched diets consumed by participants here. Additionally, the influence of exogenous ketosis on hepatic metabolism could be explored in healthy individuals consuming habitual high-carbohydrate normal-sugar diets, relevant to athletes who might utilise the KME for exercise performance or recovery. Prolonged exogenous ketosis has, for example, been shown to blunt insulin sensitivity across a one week cycling training protocol in healthy athletes[663], which may have been in part driven by accumulation of intra-hepatic palmitate[324] consequent of upregulated DNL.

Chronically elevated DNL has been linked to aberrant hepatic lipid accretion[426,572]. Future work might therefore establish if hepatic DNL still exhibits an elevated postprandial response across repeated/prolonged instances of exogenous ketosis, whilst exploring how this affects IHTAG content and composition[324]. DNL it is only one component that determines intra-hepatic TAG (IHTAG) accumulation, however. It is possible that the reduction in circulating NEFA availability for uptake into the liver, and thus storage as IHTAG, seen under exogenous ketosis may offset elevated *de novo* hepatic lipid synthesis. Chronically elevated βHB has also been shown to protect against steatosis and abnormal lipid metabolism in rodents[574,575,664,665], thus short term elevated lipogenesis might not translate to longer term pathogenesis. Investigation of chronic KME consumption may therefore better inform the potential risk of incurring detrimental outcomes associated with elevated DNL, especially in the context of increased plasma insulin[657], such as hepatic insulin resistance[324,587], fatty liver disease[311], and cardiovascular disease[666]. Reciprocally, greater insight would also be garnered regarding how these risks

might be counteracted, potentially in a population specific manner, by potential benefits of ketosis including improved glycaemic control, enhanced immune modulation[667,668], and attenuated muscle atropy[188], alongside supressed postprandial appetite, and potentially elevated HDL levels as observed in *Chapter 5*.

6.4 Concluding Remarks

Whilst work presented in this thesis does not provide evidence for exogenous ketosis augmenting exercise performance in the settings investigated, this unique phenotype nonetheless substantively modulated metabolism and physiology. It may therefore still hold ergogenic potential under conditions not explored here, such as when co-ingested with nutritional interventions known to increase blood acidity-buffering capacity. Beyond performance sport, the therapeutic potential of exogenous ketones in those with disordered metabolism may present fruitful avenues for future investigation and application. Any posited benefits may, however, need to be evaluated within the context of the metabolic impacts of ketosis that have been acutely characterised in this thesis, warranting further investigation in patient populations and across prolonged KME ingestion.

A detailed summary of the main thesis findings can be found in Appendix Q.

Appendices

Appendix A (Chapter 2)

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

Generalised Rating of Perceived Exertion (RPE) Borg[353,354] scale.

Appendix B (Chapter 2)

Breathlessness Intensity		Anxiety of Breathing		Leg Discomfort		Anxiety of Leg Discomfort	
0	No Breathlessness	0	No Breathing Anxiety	0	No Leg Discomfort	0	No Leg Discomfort Anxiety
1		1		1		1	
2		2		2		2	
3		3		3		3	
4		4		4		4	
5		5		5		5	
6		6		6		6	
7		7		7		7	
8		8		8		8	
9		9		9		9	
10	Extremely Hard Maximal Breathlessness	10	Extremely Hard Maximal Breathing Anxiety	10	Extremely Hard Maximal Leg Discomfort	10	Extremely Hard Maximal Leg Discomfort Anxiety

Localised Rating of Perceived Exertion (RPE) Scales[285]**.**

Appendix C (Chapter 2)

Gastrointestinal Symptoms Questionnaire

Please select the number on the scale (for each item) that represents the severity of your gastrointestinal symptoms at this particular moment in time

Upper abdominal problems

	None		Mild		Moderate		Severe		Unbearable
Heartburn	0	1	2	3	4	5	6	7	8
Bloating	0	1	2	3	4	5	6	7	8
Nausea	0	1	2	3	4	5	6	7	8
Vomiting	0	1	2	3	4	5	6	7	8

Lower abdominal problems

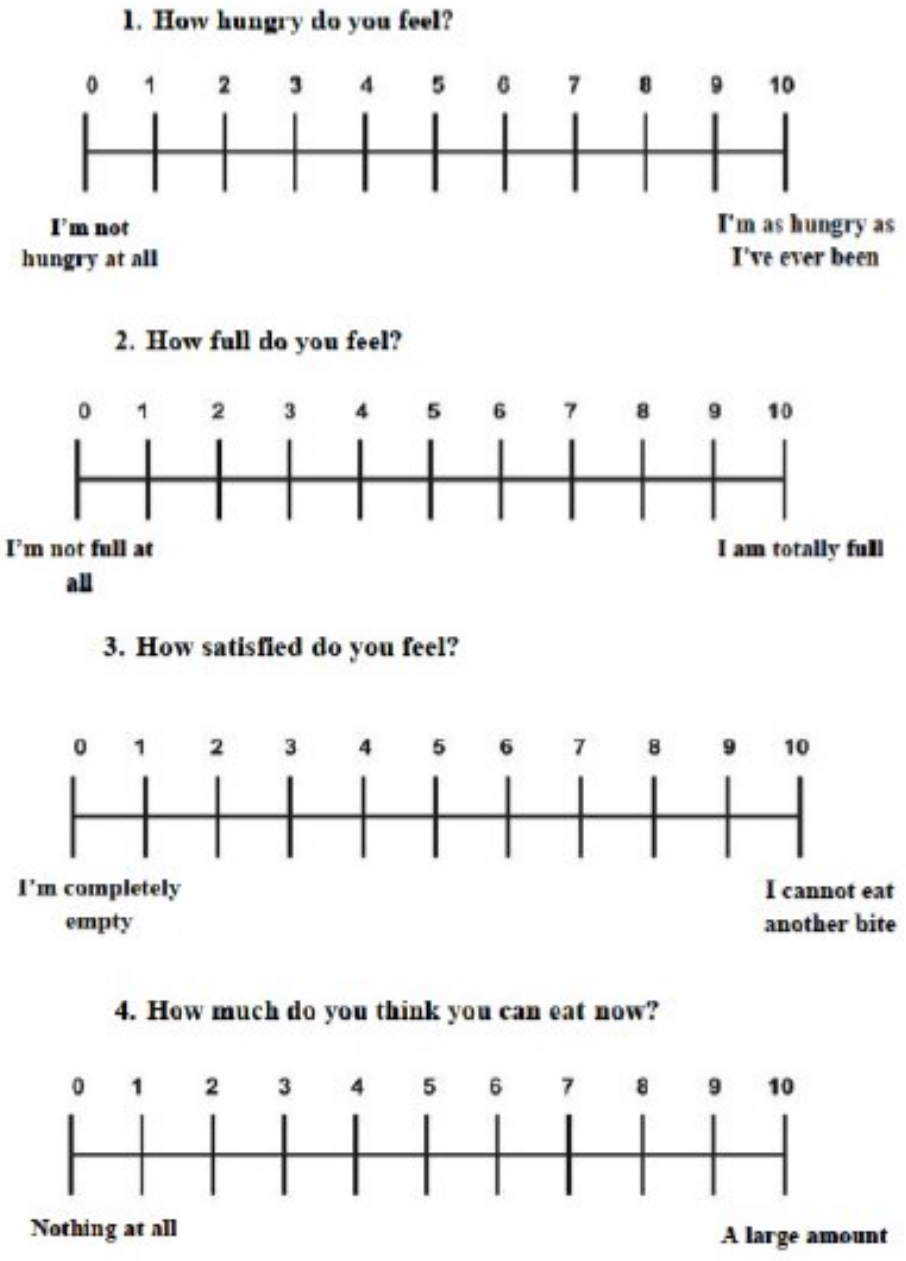
	None		Mild		Moderate		Severe		Unbearable
Intestinal cramps	0	1	2	3	4	5	6	7	8
Abdominal pain	0	1	2	3	4	5	6	7	8
Flatulence	0	1	2	3	4	5	6	7	8
Diarrhea	0	1	2	3	4	5	6	7	8

Systemic problems

	None		Mild		Moderate		Severe		Unbearable
Dizziness	0	1	2	3	4	5	6	7	8
Headache	0	1	2	3	4	5	6	7	8
Muscle cramp	0	1	2	3	4	5	6	7	8
Urge to urinate	0	1	2	3	4	5	6	7	8

Gastrointestinal (GI) Distress Scales[159,355,356]**.**

Appendix D (Chapter 2)



Appetite Scales[357]**.**

Appendix E (Chapter 3)

Resting Pilot - plasma βHB.

Time Relative to 'Exercise' Start (min)	Time Relative to Drink Consumption (min)	βHB (mM)
Drink at 1 hr Post-Breakfast		
Fasted		0.3 ± 0.2
-60	0	0.1 ± 0.1
-45	15	1.5 ± 0.3
-30	30	2.1 ± 0.4
-15	45	2.3 ± 0.3
-10	50	2.4 ± 0.4
0	60	2.5 ± 0.5
15	75	2.5 ± 0.3
30	90	2.4 ± 0.2
45	105	2.5 ± 0.3
60	120	2.3 ± 0.3
90	150	2.4 ± 0.4
Drink at 1 hr 20 min Post-Breakfast		
Fasted		0.2 ± 0.2
-40	0	0.1 ± 0.1
-20	20	1.4 ± 0.2
-10	30	1.8 ± 0.4
0	40	2.0 ± 0.3
15	55	2.2 ± 0.3
30	70	2.4 ± 0.3
45	85	2.5 ± 0.5
60	100	2.4 ± 0.4
90	130	2.5 ± 0.2
120	160	2.3 ± 0.4

Plasma βHB concentration (mM) after ingestion of 'High-KME' Drink 1 (573 mg/kg·BW KME; 8.3% glucose/maltodextrin solution). Drinks ingested either 1 hr or 1 hr 20 min after the standardised breakfast had been consumed. Participants remained at rest for the duration. Time Relative to 'Exercise' Start, refers to when the 1 hr Cycle leg would have commenced (i.e. 0 min) during exercise trials. Data presented as mean ± SEM. n = 5.

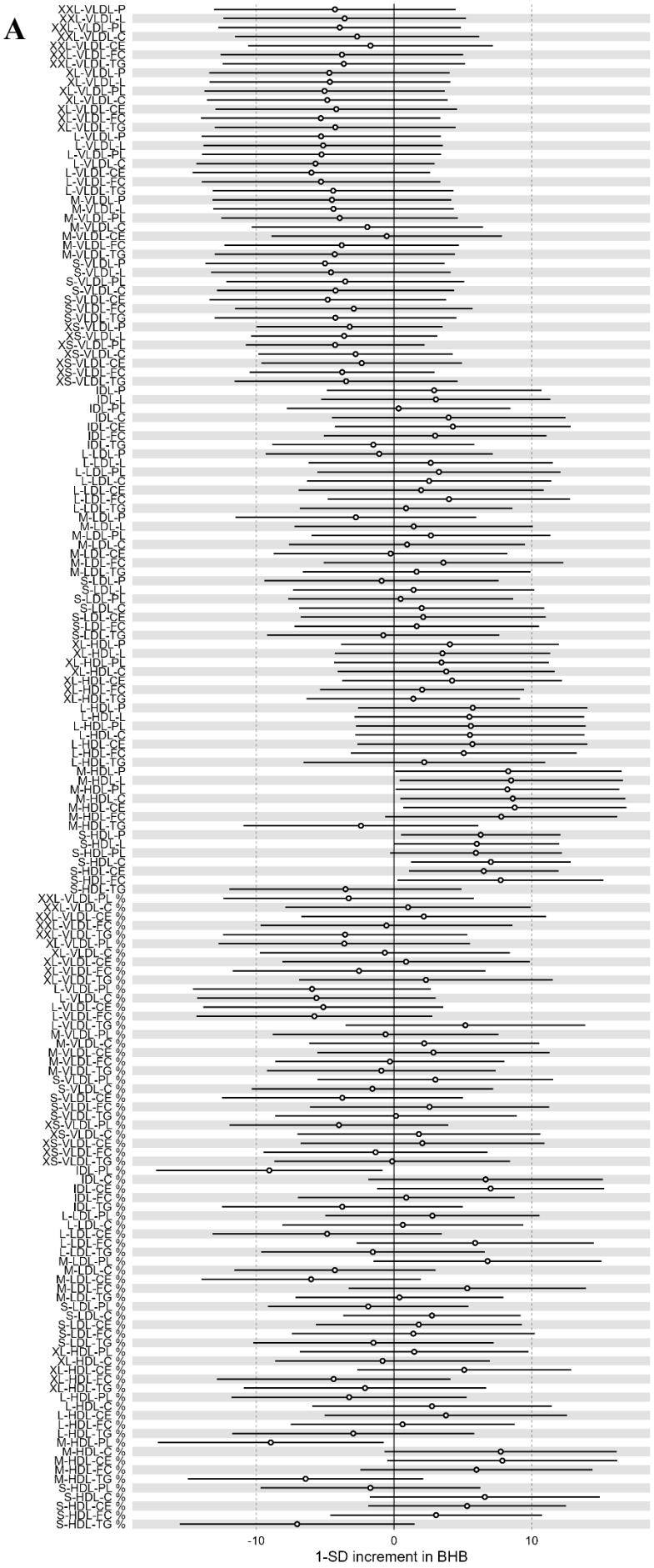
Appendix F (Chapter 3)

Fasted plasma metabolites concentrations for Placebo, Low-KME, and High-KME visits.

		High-KME			Low-KME			PLA			p Condition
Glucose	mM	5.183	±	0.141	5.128	±	0.201	5.187	±	0.083	0.763
βHB	mM	0.06	±	0.01	0.05	±	0.01	0.05	±	0.01	0.618
NEFA	mM	1.38	±	0.70	1.27	±	0.63	1.30	±	0.64	0.553
Lactate	mM	0.83	±	0.09	1.09	±	0.11	0.95	±	0.13	0.204

Data presented as Mean ± SEM. n = 7.

Appendix G (Chapter 3)



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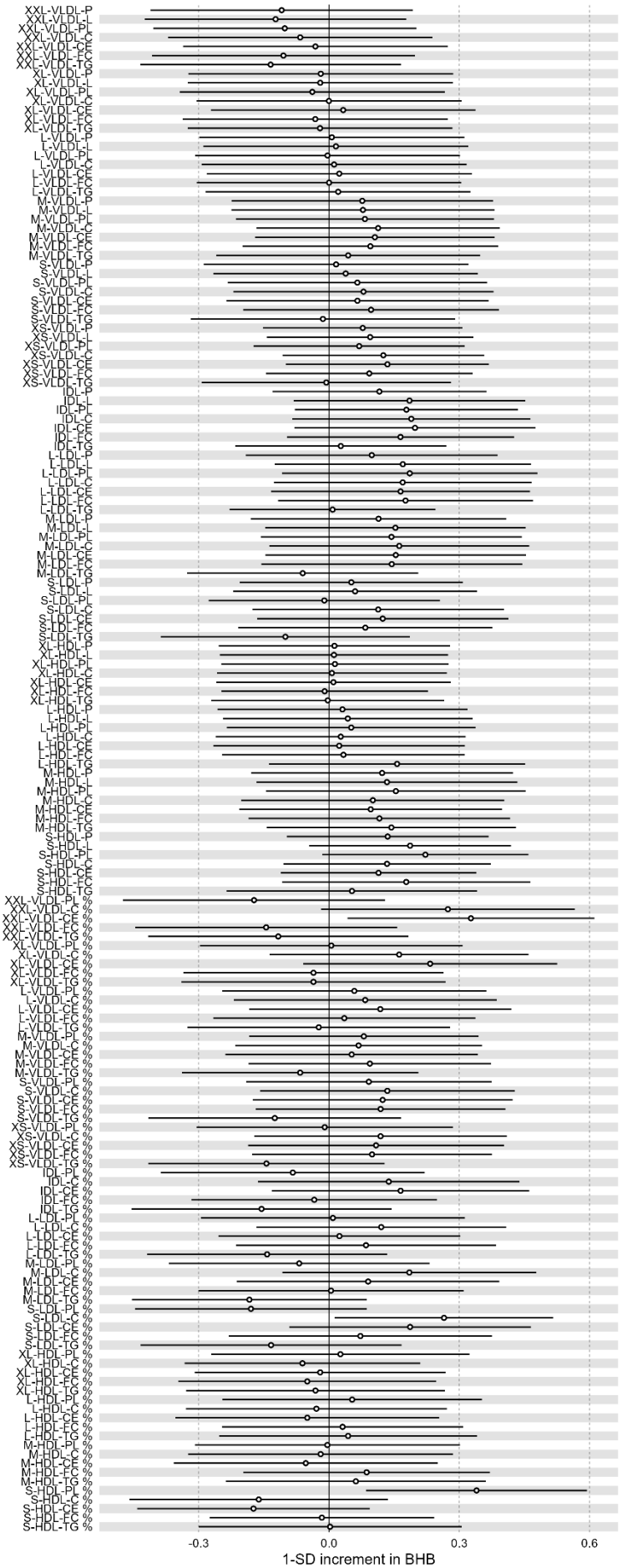
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XXL-VLDL-FC
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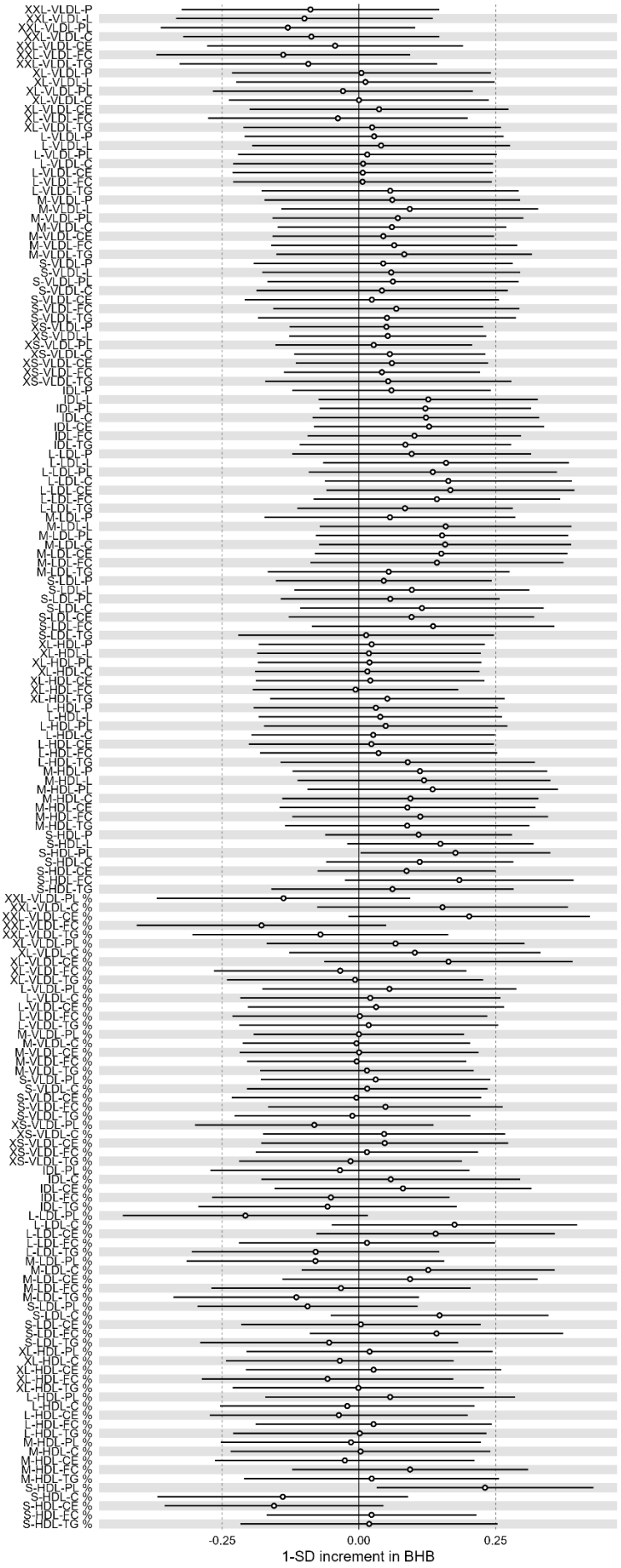
1-SD increment in BHB

C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M-LDL-PL
M-LDL-C
M-LDL-CE
M-LDL-FC
M-LDL-TG
S-LDL-P
S-LDL-L
S-LDL-PL
S-LDL-C
S-LDL-CE
S-LDL-FC
S-LDL-TG
XL-HDL-P
XL-HDL-L
XL-HDL-PL
XL-HDL-C
XL-HDL-CE
XL-HDL-FC
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L-HDL-P
L-HDL-L
L-HDL-PL
L-HDL-C
L-HDL-CE
L-HDL-FC
L-HDL-TG
M-HDL-P
M-HDL-L
M-HDL-PL
M-HDL-C
M-HDL-CE
M-HDL-FC
M-HDL-TG
S-HDL-P
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S-HDL-PL
S-HDL-C
S-HDL-CE
S-HDL-FC
S-HDL-TG
XXL-VLDL-PL %
XXL-VLDL-C %
XXL-VLDL-CE %
XXL-VLDL-FC %
XXL-VLDL-TG %
XL-VLDL-PL %
XL-VLDL-C %
XL-VLDL-CE %
XL-VLDL-FC %
XL-VLDL-TG %
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M-HDL-FC %
M-HDL-TG %
S-HDL-PL %
S-HDL-C %
S-HDL-CE %
S-HDL-FC %
S-HDL-TG %
-0.25
0.00
0.25
1-SD increment in BHB

Associations between NMR plasma lipoprotein subclasses and plasma βHB concentrations.

*Timepoints: **A**, fasted. **B**, -10 min (postprandial, post-first KME/PLA drink, resting). **C**, 60 min (postprandial, post all KME/PLA drinks, end of 1 hr cycle leg). Effect estimate is a 1-SD increase in metabolite concentration per unit increase in βHB concentration. Closed circles, significant associations (false discovery rate-corrected[395]). n = 10.*

Lipoprotein subclass abbreviations: XXL-VLDL, chylomicrons and extremely large very low density lipoprotein (VLDL; >75 nm diameter); XL-VLDL, very large VLDL (~64 nm); L-VLDL, large VLDL (~53.6 nm); M-VLDL, medium VLDL (44.5 nm); S-VLDL, small VLDL (~36.8 nm); XS-VLDL, very small VLDL (~31.3 nm); IDL, intermediate density lipoprotein (~28.6 nm); L-LDL, large low density lipoprotein (LDL; ~25.5 nm); M-LDL, medium LDL (~23 nm); S-LDL, small LDL (~18.7 nm); XL-HDL, very large high density lipoprotein (HDL; ~14.3 nm); L-HDL, large HDL (~12.1 nm); M-HDL, medium HDL (10.9 nm); S-HDL, small HDL (8.7 nm).

Measurement abbreviations: C, cholesterol; CE, cholesteryl esters; FC, free cholesterol; L, total lipids; P, particle concentration; PG, phosphoglycerides; PL, phospholipid; TG, triacylglyceride. %, ratio of specific biomarker to total lipids with a subtype.

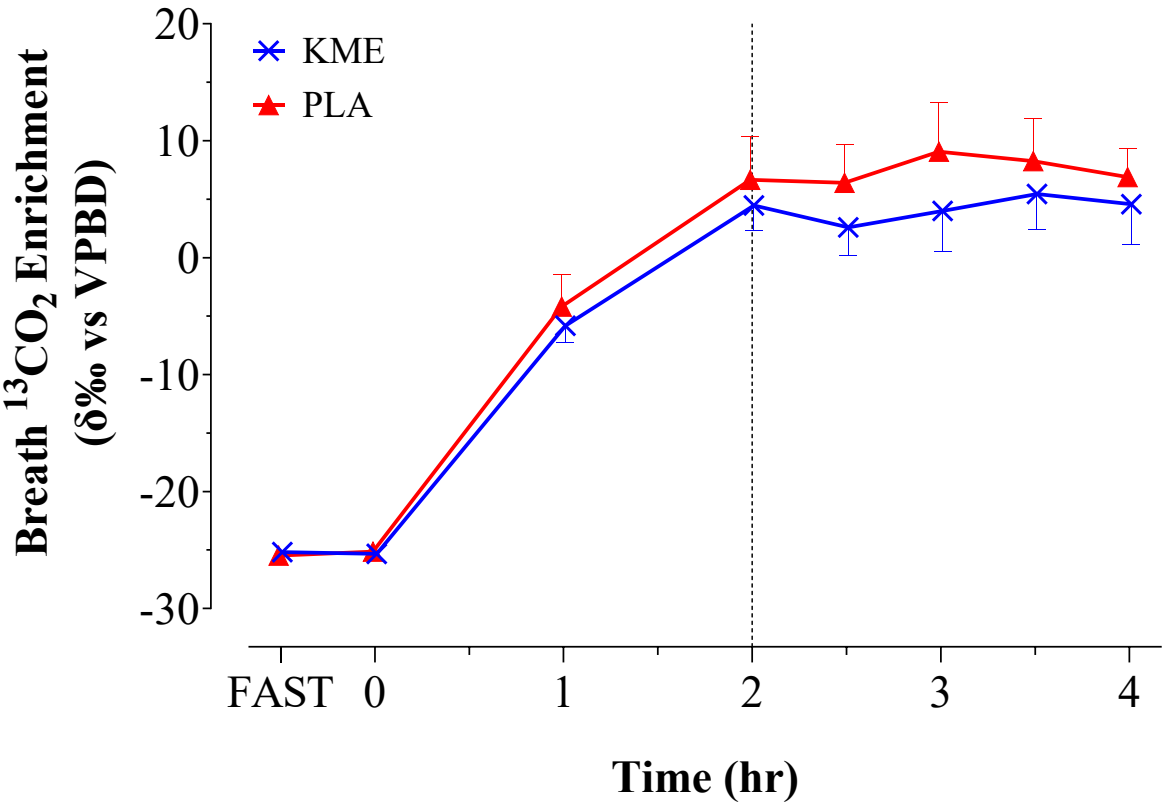
Appendix H (Chapter 4)

Fasted plasma metabolite concentrations for the KME & PLA visits.

		KME			PLA			p Condition
βHB	mM	0.039	±	0.005	0.043	±	0.004	0.254
Glucose	mM	4.79	±	0.20	4.87	±	0.32	0.669
Lactate	mM	0.76	±	0.07	0.78	±	0.07	0.679
NEFA	mM	0.38	±	0.05	0.40	±	0.03	0.755
Glycerol	µM	54	±	5	58	±	6	0.747
TAG	mM	0.55	±	0.05	0.58	±	0.04	0.455
Total Chol	mM	3.12	±	0.27	3.35	±	0.28	0.242
HDL-Chol	mM	1.37	±	0.12	1.41	±	0.11	0.534
Non-HDL-Chol	mM	1.75	±	0.16	1.94	±	0.19	0.218
ApoB	g/L	0.52	±	0.04	0.54	±	0.04	0.291
Urea	mM	5.5	±	0.3	5.2	±	0.4	0.335

Chol, cholesterol. Data presented as Mean ± SEM. p, p-value for effect of condition. n = 10.

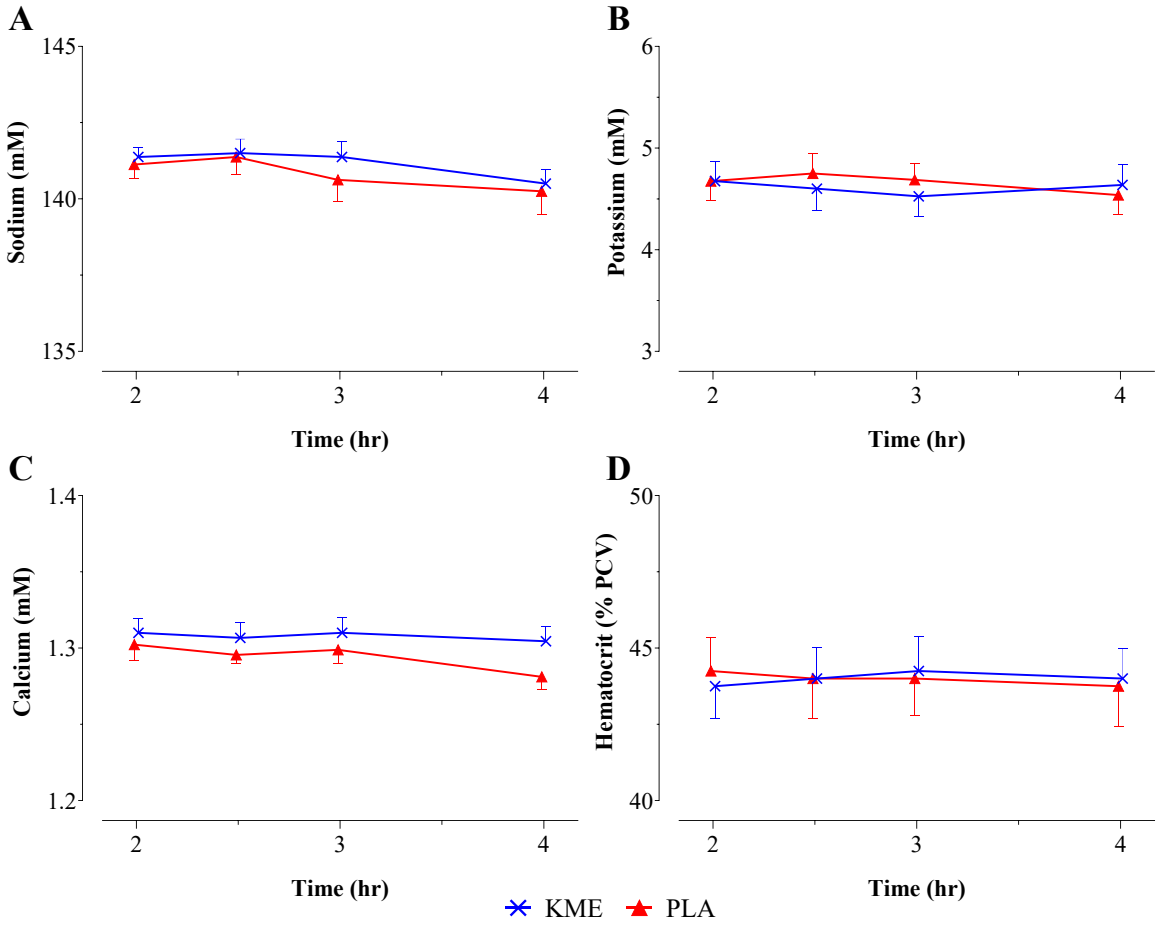
Appendix I (Chapter 4)



Expired $^{13}CO_2$ enrichment under the KME & PLA conditions.

Fasted (FAST) & across the 4 hr protocol as δ‰ vs VPBD (Vienna Pee Dee Bellemnitella). 4 hr protocol, in-exercise ingestion of U-^{13}C-glucose (0.06 g/hr) and maltodextrin (60 g/hr); FAST, Fasted timepoint. Dashed line, first KME/PLA drink (given immediately after 2 hr timepoint). Data presented as Mean ± SEM. n = 5.

Appendix J (Chapter 4)



Venous whole blood electrolyte & haematology measures during the 4 hr cycle protocol under the KME & PLA conditions.

__A__, Sodium (Na^+; mM); __B__, Potassium (K^+; mM); __C__, Calcium (Ca^{2+}; mM); __D__, Haematocrit (Hct; % packed cell volume [PCV]). First KME/PLA drink given immediately after 2 hr timepoint. Data presented as Mean ± SEM. n = 8.

Venous whole blood electrolyte & haematology measures upon completion of the TTE trial under the KME & PLA conditions.

		KME			PLA			p Condition
Sodium	mM	141	±	1	140	±	1	0.530
Potassium	mM	4.6	±	0.2	4.4	±	0.1	0.524
Calcium	mM	1.31	±	0.01	1.30	±	0.01	0.215
Haematocrit	% PCV	44	±	1	44	±	1	0.809

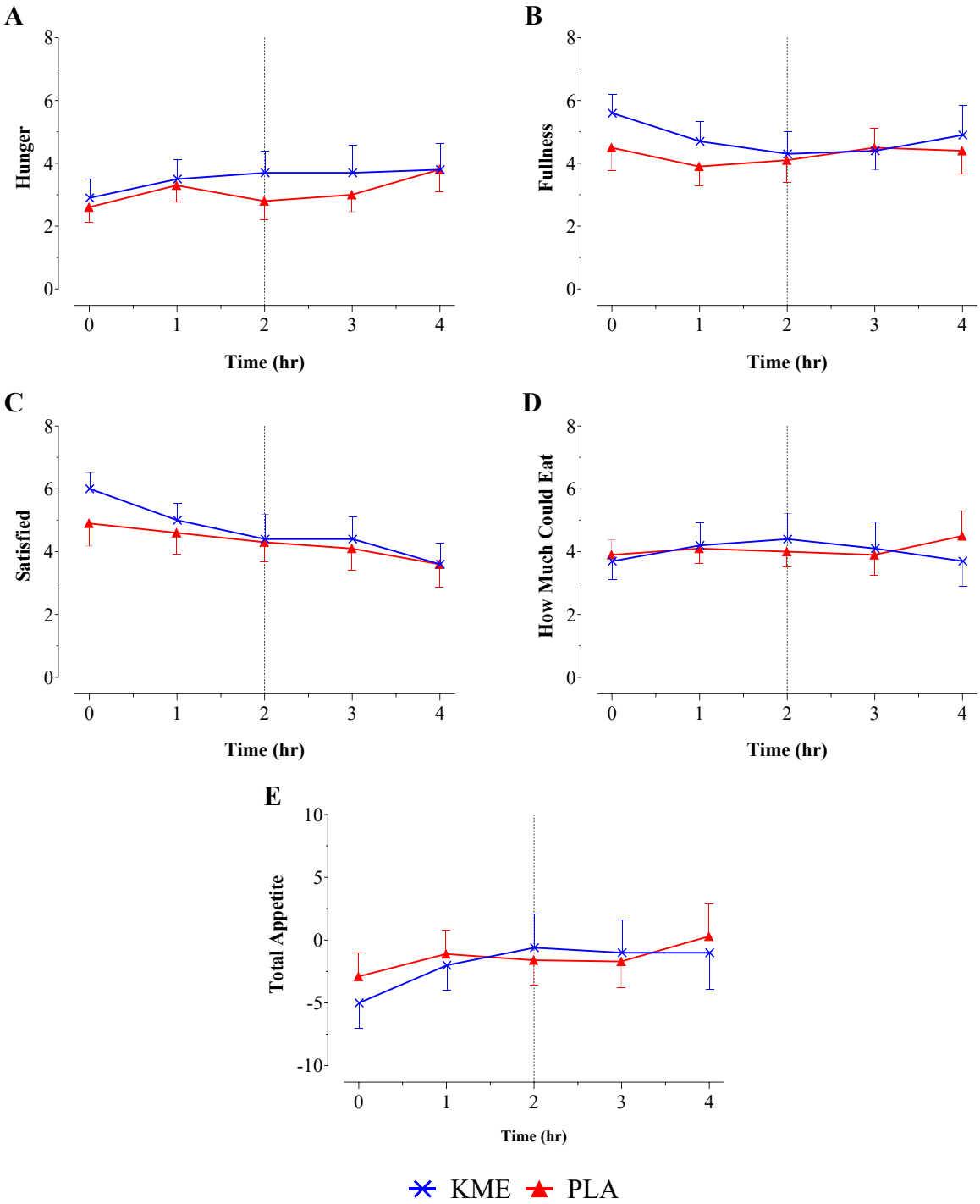
% PCV, % packed cell volume; TTE, time-to-exhaustion. Data presented as Mean ± SEM. p, p-value for effect of condition. n = 6.

Appendix K (Chapter 4)

Fasted appetite & GI distress metrics upon arrival for the KME & PLA visits.

	KME			PLA			p Condition
Hunger	6.3	±	0.4	5.8	±	0.5	0.244
Fullness	2.0	±	0.4	2.1	±	0.6	0.823
Satisfied	2.4	±	0.3	2.4	±	0.4	>0.999
How Much Could Eat	7.1	±	0.4	6.9	±	0.5	0.678
Total Appetite	9.0	±	1.1	8.2	±	1.7	0.558
GI Distress	0.3	±	0.2	0.3	±	0.2	>0.999

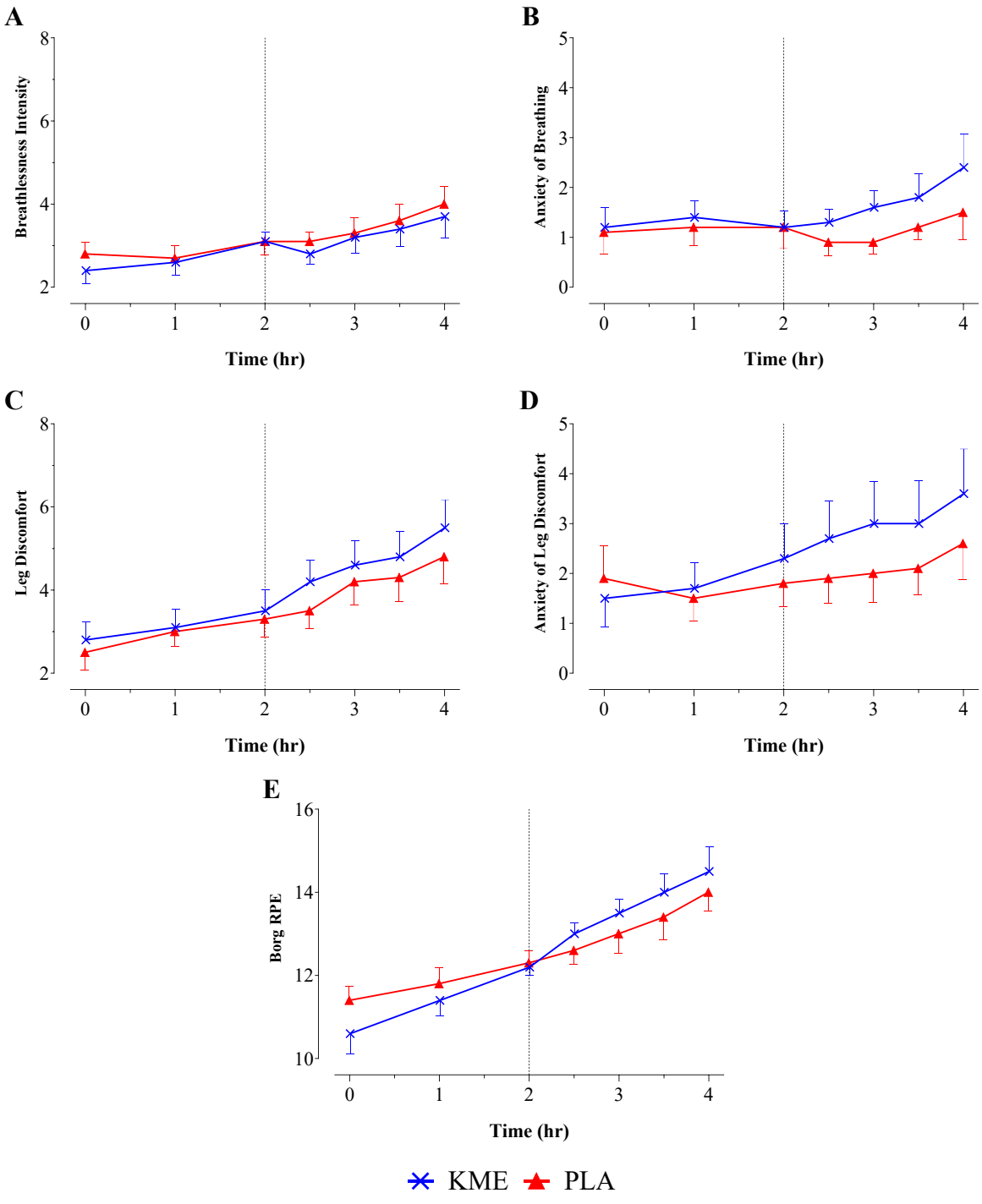
Total appetite, composite score of the four appetite metrics. GI, Gastrointestinal. Data presented as Mean ± SEM. p, p-value for effect of condition. n = 10.



Appetite metrics during the 4 hr cycle protocol under the KME & PLA conditions.

A, *Hunger;* ***B,*** *Fullness;* ***C,*** *Satisfied;* ***D,*** *How Much Could Eat;* ***E,*** *Total appetite, composite score of the four appetite metrics. Data presented as Mean ± SEM; 0-10 scale (A-D). Dashed line, first KME/PLA drink (given immediately after 2 hr timepoint). n = 10.*

Appendix L (Chapter 4)



RPE metrics during the 4 hr cycle protocol under the KME & PLA conditions.

A, *Breathlessness Intensity;* ***B,*** *Anxiety of Breathing;* ***C,*** *Leg Discomfort;* ***D,*** *Anxiety of Leg Discomfort;* ***E,*** *General Borg*[353,354] *RPE. RPE, rating of perceived exertion. Data presented as Mean ± SEM; 0-10 scale (A-D); 6-20 scale (E). Dashed line, first KME/PLA drink (given immediately after 2 hr timepoint). n = 10.*

RPE metrics upon TTE trial completion under the KME & PLA conditions.

	KME			PLA			p Condition
General Borg RPE	19.6	±	0.3	19.8	±	0.2	0.732
Breathlessness Intensity	7.8	±	0.7	8.5	±	0.5	0.142
Anxiety of Breathing	3.8	±	1.3	3.8	±	1.2	>0.999
Leg Discomfort	7.8	±	0.8	8.6	±	0.4	0.231
Anxiety of Leg Discomfort	4.0	±	1.5	2.8	±	1.1	0.160

RPE, rating of perceived exertion; TTE, time-to-exhaustion. Data presented as Mean ± SEM on a 6-20 (Borg RPE) or 0-10 (all others) scale. p, p-value for effect of condition. n = 8.

Appendix M (Chapter 5)

Example high-carbohydrate high-sugar diet.

Target kcal		3192				
Meal	**Food**	**Amount**	**Fat (g)**	**CHO (g)**	**Sugars (g)**	**Pro (g)**
Snack	Kind Protein Crunchy Peanut Butter Bars 42g	42g (1 bar)	14.0	9.7	6.7	10.0
	Bassetts Jelly Babies 190g	95g (1/2 bag)	0.0	74.1	70.3	3.3
Lunch	Tesco Tomato & Basil Pasta Salad 300g	300g (1 Pot)	15.6	120.8	26.0	21.2
	Rockstar Juiced Energy El Mango 500mL	500mL (1 can)	0.0	26.0	24.0	2.1
	Bol Mango, Passion Fruit & Coconut Power Shake 450g	450g (1 bottle)	3.3	29.7	21.2	20.6
	Warburtons 4 Soft White Pittas	1 Pitta	1.3	29.9	2.5	6.9
Snack	Kind Protein Crunchy Peanut Butter Bars 42g	42g (1 bar)	14.0	9.7	6.7	10.0
	Bassetts Jelly Babies 190g	95g (1/2 bag)	0.0	74.1	70.3	3.3
Dinner	Tesco P/Chef Meat Free Spaghetti Bolognese 450g	450g (1 pack)	9.9	69.0	10.2	20.3
	Warburtons Soft White Pittas	1 Pitta	1.3	29.9	2.5	6.9
	Tesco Sugar Snap Peas 150g	75g (1/2 bag)	0.2	3.6	2.8	3.4
	Activia Strawberry Yogurt 115g	2 Pots	6.4	28.0	28.0	9.0
	Tesco Golden Syrup 680g	2 x 15g	0.0	25.0	22.4	0.0
Total (g)			**66.0**	**529.5**	**293.6**	**117.1**
Total kcal			**3180.2**			
% Total kcal			**18.7**	**66.6**	**36.9**	**14.7**
Macronutrient information taken from www.tesco.com/groceries		***Target % Total kcal***	***20***	***65***	***32.5***	***15***

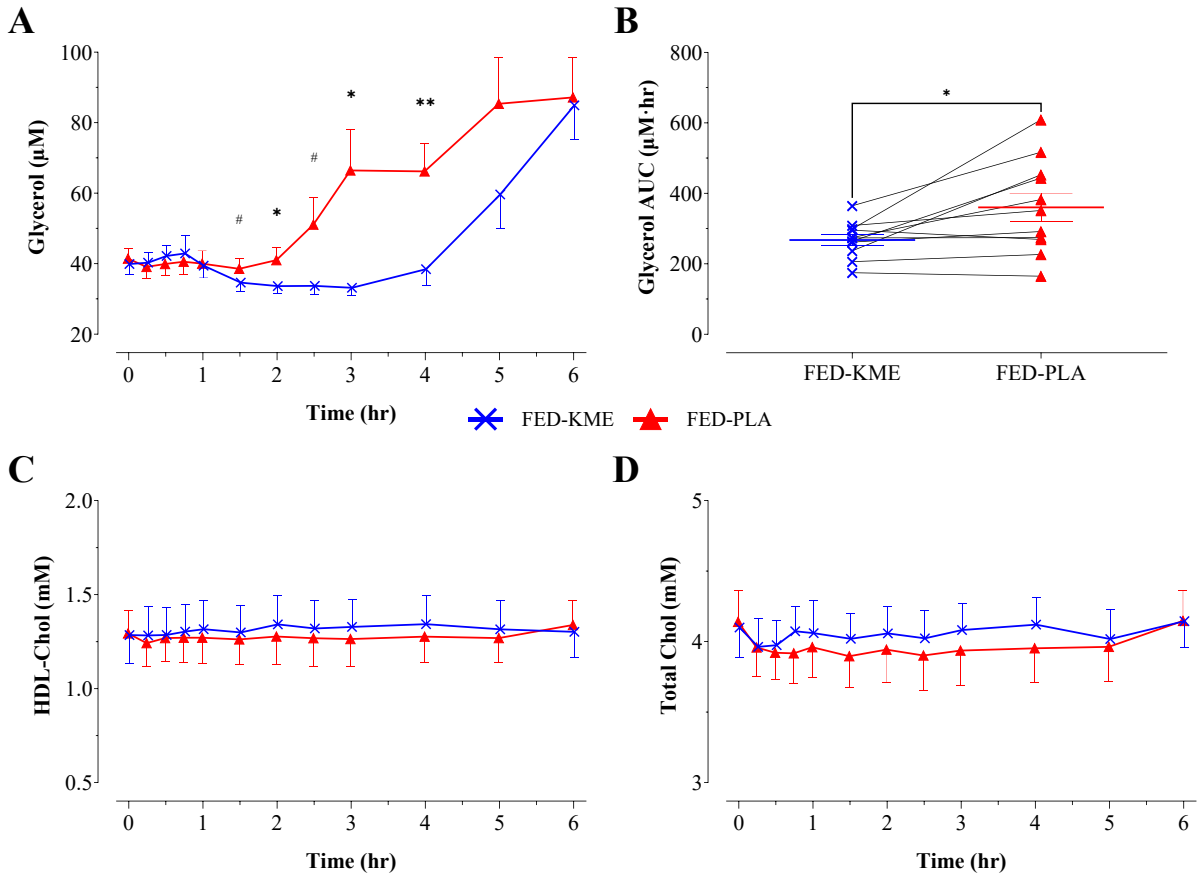
Prescribed diet shown is for one day, which was replicated for the two days prior to each Experimental visit. CHO, carbohydrate; PRO, protein.

Appendix N (Chapter 5)

Fasted plasma metabolites and breath acetone concentrations for the FED-KME & FED-PLA visits.

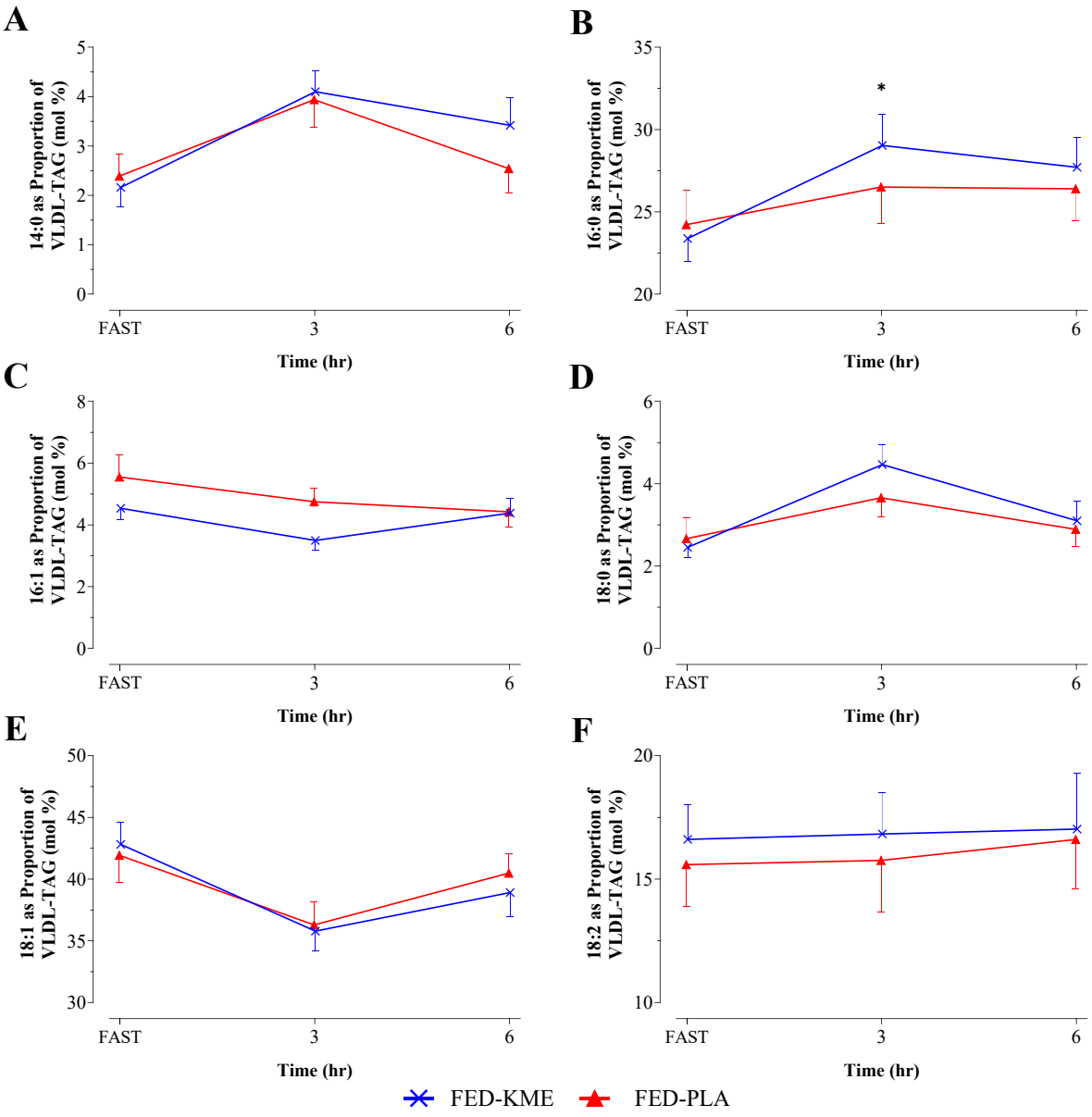
		FED-KME			FED-PLA			p Condition
βHB	mM	0.129	±	0.044	0.129	±	0.038	0.999
Glucose	mM	5.22	±	0.13	5.12	±	0.15	0.302
Lactate	mM	0.77	±	0.11	0.87	±	0.14	0.184
NEFA	mM	0.75	±	0.09	0.79	±	0.10	0.562
Glycerol	µM	73	±	6	72	±	6	0.644
TAG	mM	0.87	±	0.08	0.90	±	0.08	0.743
Total Chol	mM	4.07	±	0.15	4.03	±	0.20	0.738
HDL-Chol	mM	1.26	±	0.14	1.27	±	0.13	0.915
Non-HDL-Chol	mM	2.81	±	0.12	2.76	±	0.18	0.670
Urea	mM	5.31	±	0.45	5.32	±	0.36	0.972
Insulin	mU/L	5.89	±	1.07	5.27	±	0.93	0.601
Breath Acetone	ACE	3.9	±	0.4	3.6	±	0.3	0.434

ACE, arbitrary units; Chol, cholesterol. Data presented as Mean ± SEM. p, p-value for effect of condition. n = 11.



Plasma glycerol, HDL-cholesterol, & total cholesterol during the FED-KME & FED-PLA visits.

*__A__, glycerol concentration (µM); __B__, glycerol AUC (µM·hr); __C__, HDL-chol concentration (mM); __D__, total chol concentration (mM). AUC, area-under-the-curve; chol, cholesterol. Data presented as Mean ± SEM. # $p < 0.05$, * $p < 0.05$, ** $p < 0.01$ between FED-KME and FED-PLA conditions. n = 11.*



Fatty acid proportion of VLDL-TAG during the FED-KME & FED-PLA visits.

*__A__, 14:0 (mol%); __B__, 16:0 (mol%; exploratory post-hoc analyses); __C__, 16:1 (mol%); __D__, 18:0 (mol%; exploratory post-hoc analyses); __E__, 18:1 (mol%); __F__, 18:2 (mol%). Data presented as Mean ± SEM. __*__ $p < 0.05$ between FED-KME and FED-PLA conditions. $n = 10$.*

Baseline (FAST) concentrations did not differ between conditions for any VLDL-TAG fatty acid ($p \geq 0.170$).

Respiratory measures across the FED-KME & FED-PLA visits.

	Timepoint	FED-KME	FED-PLA	p Time	p Condition	p Interaction
$\dot{V}O_2$ (L/min)	FASTED	0.28 ± 0.01	0.28 ± 0.02	<0.001	0.746	0.773
	60 min	0.32 ± 0.02	0.33 ± 0.02			
	300 min	0.29 ± 0.01	0.28 ± 0.01			
$\dot{V}CO_2$ (L/min)	FASTED	0.24 ± 0.01	0.24 ± 0.01	<0.001	0.893	0.888
	60 min	0.28 ± 0.02	0.29 ± 0.01			
	300 min	0.24 ± 0.01	0.23 ± 0.01			
RER	FASTED	0.84 ± 0.02	0.85 ± 0.01	0.004	0.704	0.971
	60 min	0.88 ± 0.01	0.88 ± 0.01			
	300 min	0.82 ± 0.01	0.83 ± 0.01			
BF (/min)	FASTED	15.3 ± 0.9	16.0 ± 0.9	0.004	0.808	0.819
	60 min	17.2 ± 1.0	17.1 ± 0.7			
	300 min	16.1 ± 1.0	16.2 ± 0.7			
V_T (L)	FASTED	0.59 ± 0.04	0.54 ± 0.03	0.016	0.331	0.567
	60 min	0.63 ± 0.04	0.61 ± 0.03			
	300 min	0.58 ± 0.03	0.54 ± 0.03			
V_E (L/min)	FASTED	8.76 ± 0.40	8.56 ± 0.63	<0.001	0.367	0.914
	60 min	10.78 ± 0.86	10.26 ± 0.44			
	300 min	9.19 ± 0.74	8.60 ± 0.57			

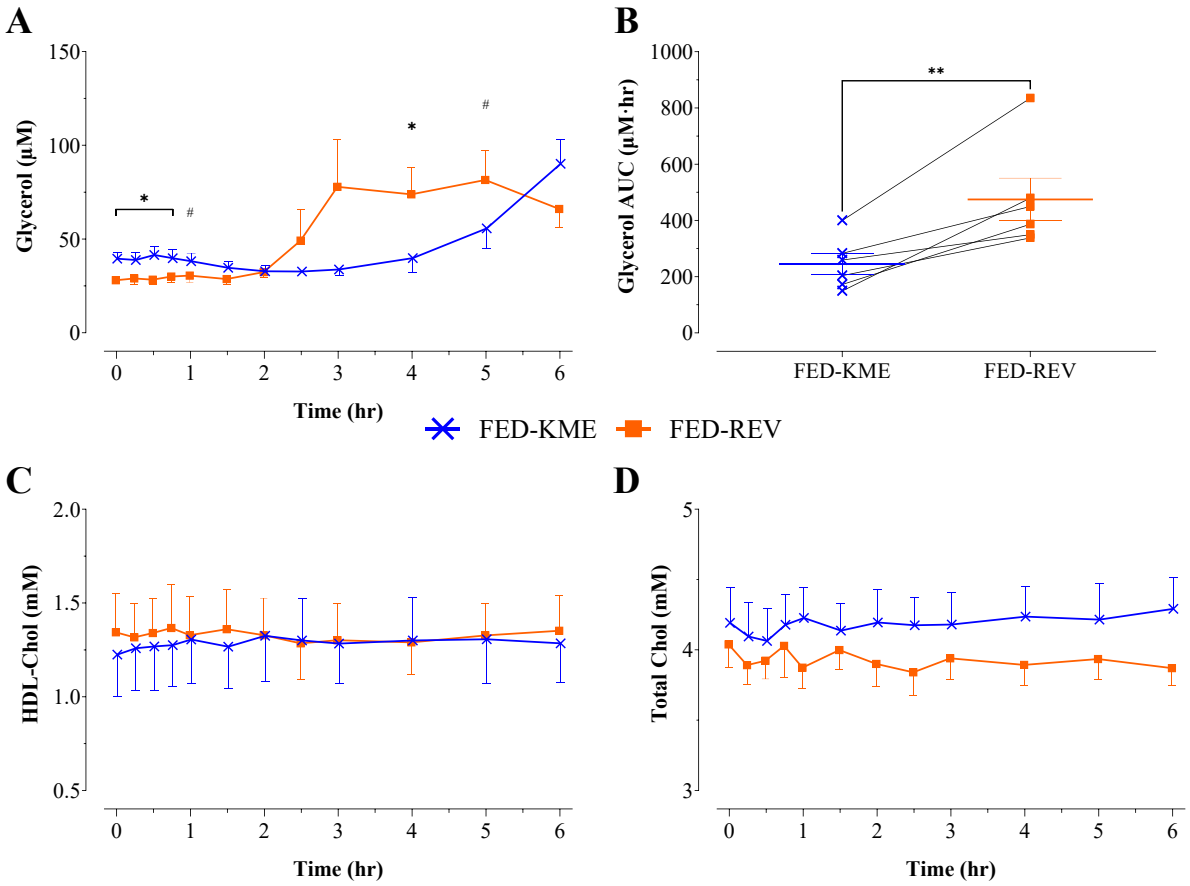
$\dot{V}O_2$, oxygen utilisation (L/min); $\dot{V}CO_2$, carbon dioxide production (L/min); RER, respiratory exchange ratio; BF, breathing frequency (/min); V_T, tidal volume (L); V_E, minute ventilation (L/min). p, p-value for given main/interaction effect. Data presented as Mean ± SEM. n = 11.

Appendix O (Chapter 5)

Fasted plasma metabolite and breath acetone concentrations for the FED-KME & FED-REV visits.

		FED-KME			FED-REV			p Condition
βHB	mM	0.090	±	0.012	0.078	±	0.014	0.555
Glucose	mM	5.36	±	0.19	5.25	±	0.17	0.447
Lactate	mM	0.73	±	0.11	0.68	±	0.11	0.776
NEFA	mM	0.83	±	0.15	0.87	±	0.14	0.623
Glycerol	μM	72	±	9	65	±	11	0.664
TAG	mM	1.03	±	0.09	1.07	±	0.08	0.675
Total Chol	mM	4.13	±	0.18	4.03	±	0.17	0.213
HDL-Chol	mM	1.21	±	0.23	1.27	±	0.18	0.700
Non-HDL-Chol	mM	2.92	±	0.17	2.78	±	0.13	0.320
Insulin	mU/L	6.00	±	1.36	5.58	±	1.50	0.364
Breath Acetone	ACE	4.2	±	0.3	4.0	±	0.2	0.695

ACE, arbitrary units; Chol, cholesterol. Data presented as Mean ± SEM. p, p-value for effect of condition. n = 6.



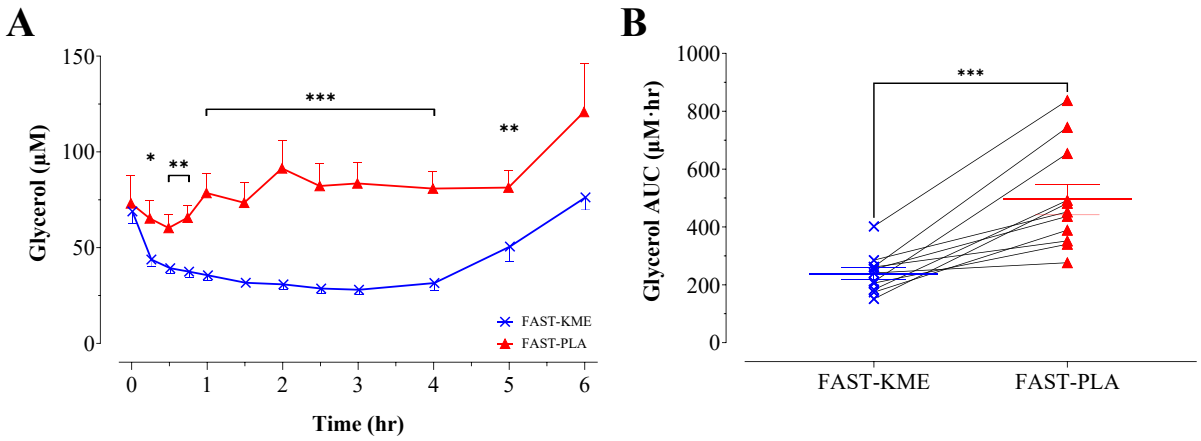
Plasma glycerol, HDL-cholesterol, & total cholesterol during the FED-KME & FED-REV visits. *__A__, glycerol concentration (µM); __B__, glycerol AUC (µM·hr); __C__, HDL-chol concentration (mM); __D__, total chol concentration (mM). AUC, area-under-the-curve; chol, cholesterol. Data presented as Mean ± SEM. # $p < 0.05$, * $p < 0.05$, ** $p < 0.01$ between FED-KME and FED-PLA conditions. n = 6.*

Respiratory measures across the FED-KME & FED-REV visits.

	Timepoint	FED-KME	FED-REV	p Time	p Condition	p Interaction
$\dot{V}O_2$ (L/min)	FASTED	0.30 ± 0.01	0.31 ± 0.02	0.018	0.109	0.548
	60 min	0.36 ± 0.02	0.37 ± 0.02			
	300 min	0.30 ± 0.01	0.30 ± 0.02			
$\dot{V}CO_2$ (L/min)	FASTED	0.25 ± 0.01	0.26 ± 0.02	0.014	0.184	0.645
	60 min	0.31 ± 0.02	0.32 ± 0.02			
	300 min	0.24 ± 0.01	0.25 ± 0.02			
RER	FASTED	0.83 ± 0.02	0.83 ± 0.01	0.017	0.970	0.593
	60 min	0.87 ± 0.01	0.86 ± 0.01			
	300 min	0.82 ± 0.01	0.82 ± 0.01			
BF (/min)	FASTED	14.7 ± 0.9	13.7 ± 0.97	0.042	0.323	0.591
	60 min	17.1 ± 1.0	16.8 ± 1.06			
	300 min	16.6 ± 1.1	16.5 ± 0.86			
V_T (L)	FASTED	0.57 ± 0.03	0.59 ± 0.02	0.043	0.797	0.419
	60 min	0.67 ± 0.04	0.69 ± 0.04			
	300 min	0.56 ± 0.02	0.55 ± 0.03			
V_E (L/min)	FASTED	8.38 ± 0.50	8.11 ± 0.77	0.032	0.198	0.818
	60 min	11.39 ± 0.99	11.35 ± 0.88			
	300 min	9.27 ± 0.86	8.90 ± 0.64			

$\dot{V}O_2$, oxygen utilisation (L/min); $\dot{V}CO_2$, carbon dioxide production (L/min); RER, respiratory exchange ratio; BF, breathing frequency (/min); V_T, tidal volume (L); V_E, minute ventilation (L/min). p, p-value for given main/interaction effect. Data presented as Mean ± SEM. n = 6.

Appendix P (Chapter 5)



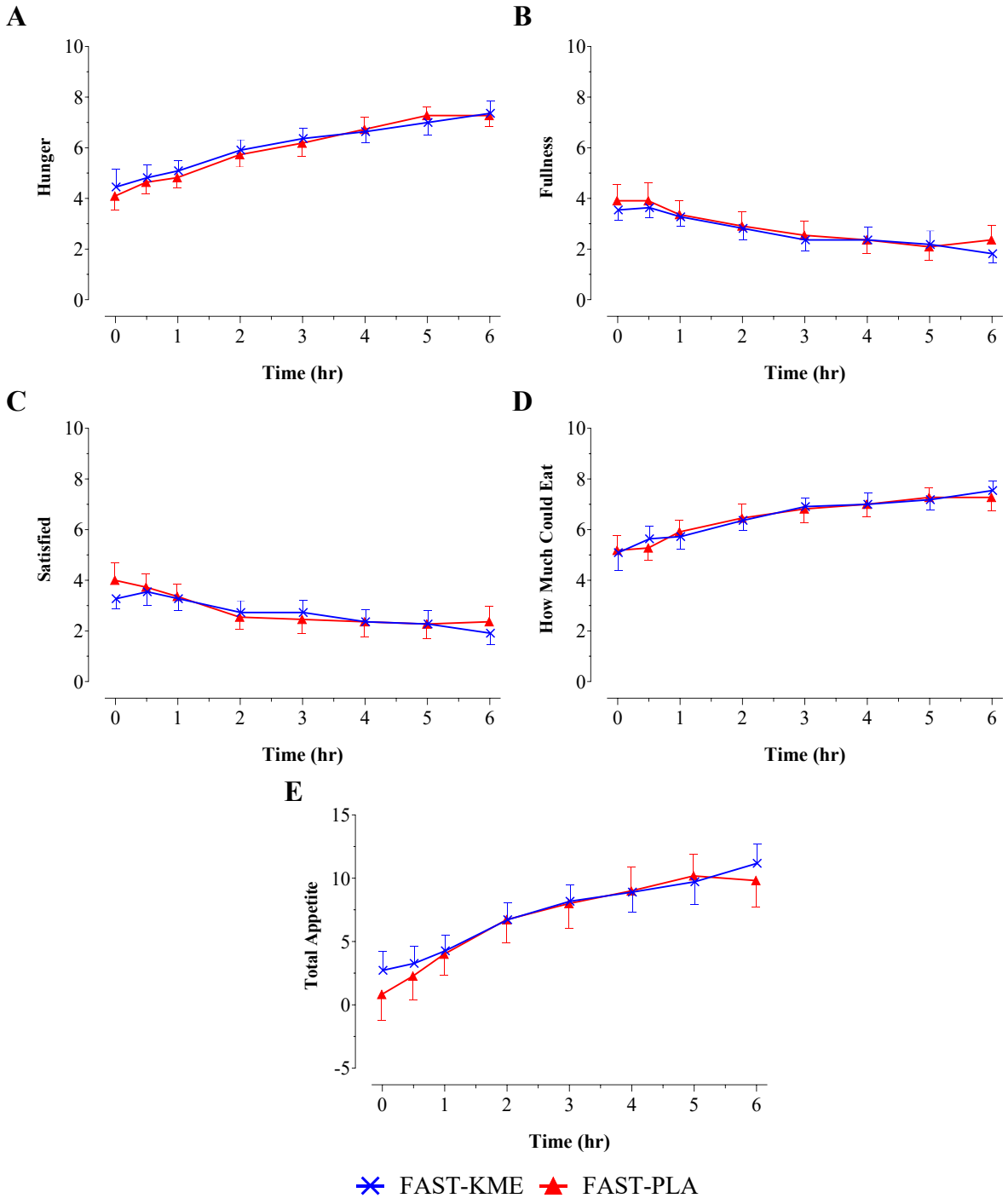
Plasma glycerol during the FAST-KME & FAST-PLA visits.

***A**, glycerol concentration (µM); **B**, glycerol AUC (µM·hr). AUC, area-under-the-curve. Data presented as Mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ between FAST-KME and FAST-PLA conditions. $n = 11$.*

Respiratory measures across the FAST-KME & FAST-PLA visits.

	Timepoint	FAST-KME	FAST-PLA	p Time	p Condition	p Interaction
$\dot{V}O_2$ (L/min)	FASTED	0.28 ± 0.02	0.28 ± 0.02	0.209	0.282	0.375
	60 min	0.27 ± 0.02	0.29 ± 0.02			
	300 min	0.27 ± 0.02	0.27 ± 0.02			
$\dot{V}CO_2$ (L/min)	FASTED	0.23 ± 0.02	0.24 ± 0.01	0.011	0.318	0.726
	60 min	0.22 ± 0.01	0.23 ± 0.01			
	300 min	0.22 ± 0.01	0.22 ± 0.01			
RER	FASTED	0.83 ± 0.01	0.85 ± 0.01	0.099	0.511	0.168
	60 min	0.83 ± 0.03	0.82 ± 0.02			
	300 min	0.80 ± 0.01	0.81 ± 0.01			
BF (/min)	FASTED	16.3 ± 0.8	16.3 ± 0.6	0.221	0.425	0.639
	60 min	15.4 ± 1.0	16.3 ± 0.8			
	300 min	15.1 ± 1.0	15.8 ± 0.9			
V_T (L)	FASTED	0.51 ± 0.03	0.53 ± 0.03	0.233	0.632	0.401
	60 min	0.57 ± 0.04	0.54 ± 0.02			
	300 min	0.55 ± 0.04	0.53 ± 0.03			
V_E (L/min)	FASTED	8.25 ± 0.56	8.64 ± 0.61	0.036	0.570	0.189
	60 min	8.59 ± 0.52	8.70 ± 0.55			
	300 min	8.13 ± 0.63	8.21 ± 0.59			

$\dot{V}O_2$, oxygen utilisation (L/min); $\dot{V}CO_2$, carbon dioxide production (L/min); RER, respiratory exchange ratio; BF, breathing frequency (/min); V_T, tidal volume in litres (L); V_E, minute ventilation (L/min). p, p-value for given main/interaction effect. Data presented as Mean ± SEM. n = 11.



Appetite metrics across the FAST-KME & FAST-PLA visits.

A, *Hunger;* ***B,*** *Fullness;* ***C,*** *Satisfied;* ***D,*** *How Much Could Eat;* ***E,*** *Total appetite, composite score of the four appetite metrics. Data presented as Mean ± SEM; 0-10 scale (A-D). n = 11.*

Appendix Q (Chapter 6)

Chapter 3

Aim: To investigate if ingestion of the ketone monoester (KME), in a dose-response manner, elicits multimodal endurance exercise performance benefits in a high-carbohydrate postprandial setting.

- Plasma βHB was elevated in a dose dependant manner to ~2.1-2.6 mM and ~0.7-1.5 mM after ingestion of 859.5 mg/kg·BW (BW, bodyweight; High-KME) or 573 mg/kg·BW (Low-KME) of the ketone monoester (KME), respectively.
- Neither High-KME nor Low-KME impacted 10 km running time trial (TT) performance, assessed immediately after cycling for 1 hr at 70% $\dot{V}O_{2peak}$ power and undertaken in a high-carbohydrate postprandial state, compared to the placebo condition (PLA).
- At rest and in-exercise, plasma glucose-AUC was 17.1% lower under High-KME compared to PLA, whilst lactate-AUC in the High-KME condition was decreased by 15.7% and 27.2% compared to Low-KME and PLA, respectively.
- Circulating βHB concentration was positively associated with plasma glutamine concentration and the degree of fatty acid (FA) saturation, both at rest and in-exercise, whilst glucagon-like peptide 1 (GLP-1) and peptide YY (PYY) levels were lower under High-KME compared to PLA.

Chapter 4

Aim: To determine if exogenous ketosis influences cycling economy, and subsequent high intensity work capacity, whilst in a glycogen depleted (low-carbohydrate) state achieved through prolonged moderate intensity exercise.

- After exogenous ketosis was achieved through KME ingestion (1000 mg/kg·BW; plasma βHB ~2.5 mM) from 2 hr onwards during 4 hr moderate intensity cycling, gross economy was unaffected, but delta economy was worsened by 10.4%, compared to the PLA condition.
- RER remained ≥0.9 under KME from 2-4 hr, whereas it was seen to progressively decline in PLA, indicating a proportionally lesser contribution of FA oxidation to energy expenditure during ketosis.
- Exogenous carbohydrate oxidation AUC across 2-4 hr was reduced by 8.3% in KME, whilst plasma glucose and non-esterified fatty acid (NEFA) concentrations were supressed.
- Blood pH was lowered by ~0.06 under KME for 2-4 hr, with accompanying depletion of circulating HCO_3^- stores, whilst V_E was increased by 6.36 L/min.
- High intensity work capacity proceeding this 4 hr protocol, assessed as time-to-exhaustion (TTE) at non-fatigued VT2 power, was unaffected by condition.
- Plasma lactate at the end of the TTE was supressed by 1.09 mM during KME compared to PLA, whilst $\dot{V}CO_2$ and heart rate were both lower across the TTE test duration.

Chapter 5

Aim: To examine whether exogenous ketosis modulates postprandial hepatic de novo lipogenesis (DNL) in healthy individuals, and to characterise how KME ingestion influences circulating metabolism in fed and fasted states.

- Postprandial hepatic DNL was elevated by 49.0% at 3 hr, and 76.4% at 6 hr (relative % change), after 573 mg/kg·BW KME ingestion compared to a non-caloric PLA, though plasma very low density lipoprotein-triacylglycerol (VLDL-TAG) concentration did not differ between conditions.
- Insulin-AUC was 33.7% and 35.5% greater, postprandially and fasted respectively, during exogenous ketosis compared to PLA, whilst ketosis increased plasma lactate, and reduced glucose and NEFA, levels in both nutritional states.
- KME ingestion increased high density lipoprotein (HDL) cholesterol concentration by ~0.1 mM compared to PLA in the fasted state only.
- Plasma βHB C_{max} was ~0.9 mM, and βHB-AUC 21.9%, lower when ingestion of the KME proceeded a high-carbohydrate meal compared to when the same dose was ingested fasted, with T_{max} not differing between the nutritional states.

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