Effect of acute ingestion of exogenous ketone supplements on exercise metabolism, physical and cognitive performance in athletes

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Mark Evans

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Abstract

Ketone bodies, namely beta-hydroxybutyrate (βHB), acetoacetate (AcAc) and acetone, are produced in the liver during physiological states and manipulations that result in reduced carbohydrate availability. Exogenous ketone supplements, namely ketone esters and ketone salts, have been developed with the aim of achieving acute nutritional ketosis i.e. βHB concentrations > 0.5 mM. We investigated whether exogenous ketone supplements had an effect on the metabolic response to exercise, physical and cognitive performance in athletes.

A literature review was undertaken to examine the relationship between elevated ketone bodies, achieved via intravenous fusion of ketone bodies or fasting, and metabolism both at rest and during exercise[1]. The review focused on seminal work performed in the 1970s and 1980s and identified a number of metabolic effects that may have relevance to improve performance and recovery in athletic populations.

Study 1 investigated the effect of a commercially-available ketone salt product formulation on the metabolic response to a graded exercise session in trained endurance athletes[2]. We observed an elevation in βHB concentrations (0.4-0.5 mM), a reduction in plasma glucose concentrations, but no effect on plasma lactate concentrations or exercise efficiency.

Study 2 investigated the effect of co-ingestion with carbohydrate of a ketone ester supplement in the form of a (R)-3-hydroxybutyl (R)-3-hydroxybutyrate ketone mono ester on

physical and cognitive performance in team sport athletes in response to a simulated soccer task[3]. Ingestion of the ketone ester had no effect on 15 m sprint times during the simulated task, or on reaction time or sustained attention performed afterwards. Compared to carbohydrate alone, we observed a preservation in executive function, measured by a decision making task, but a possible impairment in performance in a short high intensity intermittent performance test in the ketone ester condition.

Study 3 investigated the effect in trained runners of co-ingestion with carbohydrate of a (R)-3-hydroxybutyl (R)-3-hydroxybutyrate ketone mono ester on the metabolic response to submaximal exercise, and performance in 10 km time trial and cognitive tasks. We observed no effect on endurance performance or cognitive performance with the ketone ester compared to the carbohydrate alone condition.

Future research should focus on exploring the optimal dosage and timing of exogenous ketone supplements around exercise to confer performance benefits, if any. It remains to be confirmed which exercise modalities may benefit from exogenous ketone supplementation.

References

1. **Evans M**, Cogan KE, Egan B (2017) Metabolism of ketone bodies during exercise and training: physiological basis for exogenous supplementation. *Journal of Physiology* 595(9):2857–71.

2. **Evans M**, Patchett E, Nally R, Kearns R, Larney M, Egan B (2018) Effect of acute ingestion of A-hydroxybutyrate salts on the response to graded exercise in trained cyclists. *European Journal of Sport Science* 18(3):376–86.

3. **Evans M**, Egan B (2018). Intermittent running and cognitive performance after ketone ester ingestion. *Medicine & Science in Sports & Exercise* 50(11):2330-2338.

Peer-reviewed Publications Arising from this Thesis

Literature review

Published in the Journal of Physiology in May 2017 (appendix A).

Evans M, Cogan KE, Egan B (2017) Metabolism of ketone bodies during exercise and training: physiological basis for exogenous supplementation. *Journal of Physiology* 595(9):2857–71.

Original research article

Published in the European Journal of Sport Science in April 2018 (appendix B).

Evans M, Patchett E, Nally R, Kearns R, Larney M, Egan B (2018) Effect of acute ingestion of β-hydroxybutyrate salts on the response to graded exercise in trained cyclists. *European Journal of Sport Science* 18(3):376–86.

Original research article

Published in Medicine and Science in Sports and Exercise in November 2018 (appendix C).

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Original research article

Published in Medicine and Science in Sports and Exercise in November 2019 (Appendix D)

Evans M, McSwiney FT, Brady AJ (2019). No benefit of ingestion of a ketone mono ester supplement on 10-km running performance. *Medicine & Science in Sports & Exercise doi: 10.1249/MSS.0000000000002065*

Conference Proceedings Arising from this Thesis

Oral presentation

European College of Sport Science annual meeting, Vienna 2016.

Ingestion of the ketone body beta-hydroxybutyrate alters the metabolic response to exercise in trained cyclists.

Poster presentation

Cell Symposia, Gothenburg 2017.

Ingestion of the ketone body beta-hydroxybutyrate alters the metabolic response to exercise in trained cyclists.

Oral presentation

European College of Sport Science annual meeting, Dublin 2018.

Intermittent running and cognitive performance after ketone ester ingestion.

Invited speaker

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Exogenous ketone supplements: effects on exercise metabolism and performance.

Additional Peer-reviewed Publications Independent of this Thesis

Original research article

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Evans M, Tierney P, Gray N, Hawe G, Macken M, Egan B (2018) Acute ingestion of caffeinated chewing gum improves repeated sprint performance of team sport athletes with low habitual caffeine consumption. *International Journal of Sport Nutrition and Exercise Metabolism* 28(3):221-227.

Original research article

Published in the European Journal of Applied Physiology in February 2018.

Cogan KE, **Evans M,** Iuliano E, Melvin A, Susta D, Neff K, De Vito G, Egan B (2018) Co-ingestion of protein or a protein hydrolysate with carbohydrate enhances anabolic signaling, but not glycogen resynthesis, following recovery from prolonged aerobic exercise in trained cyclists. *European Journal of Applied Physiology* 118(2):349-359.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

3-hydroxybutyrase dehydrogenase	BDH
Acetoacetate	AcAc
Acetoacetyl coenzyme-A	AcAc-CoA
Acetyl coenzyme-A acetyltransferase	ACAT
Area under the curve	AUC
ATP-phosphocreatine system	PCr
Adenosine tri-phosphate	ATP
Acetyl coenzyme-A	Ac-CoA
Acetyl-CoA carboxylase	ACC
Beats per minute	bpm
Body mass index	BMI
beta-hydroxybutyrate	βHB
βHB salts condition	KET
Calorie	kcal
Calorie per kilogram	$kcal.kg^{-1}$
Calorie per minute	$kcal.min^{-1}$
Carbohydrate	CHO
Carbon dioxide	CO_2
Carbon dioxide production	$\dot{V}CO_2$
Tricarboxylic acid cycle	TCA
Delta efficiency	DE
Dry weight	DW
Dual-energy X-ray absorptiometry	DXA
D-βeta-hydroxybutyrate	DβHB
Effect size	ES
Free fatty acids	FFA
Gastrointestinal	GI
Gram	g
Gram per hour	$g.h^{-1}$
Gram per kilogram body mass	$g.kg^{-1}$ body mass
Gram per kilogram body mass per hour	$g.kg^{-1}$ body mass$.h^{-1}$
Gram per minute	$g.min^{-1}$
Gross efficiency	GE
Heart rate	HR
High-intensity interval training	HIIT
Histone acetyltransferases	HAT
Histone deacetylase	HDAC
Hour	h
Hydroxymethylglutaryl-CoA synthase	HMGCS
Intramuscular triglyceride	IMTG
Joule	J
Ketone bodies	KB
Ketone ester condition	KE
Ketone monoester plus carbohydrate condition	CHO+KME
Ketone salts	KS

Kilogram	kg
Kilojoule	kJ
Kilojoule per minute	$kJ.min^{-1}$
Kilometre	km
Kilometre per hour	$km.h^{-1}$
L-βeta-hydroxybutyrate	LβHB
Loughborough intermittent shuttle test	LIST
Mammalian target of rapamycin	mTOR-1
Maximal oxygen consumption	$\dot{V}O_{2peak}$
Maximum heart rate	HR_{max}
Medium chain triglycerides	MCT
Meter	m
Milligram	mg
Milligram per kilogram body mass	$mg.kg^{-1}$ body mass
Millilitre	mL
Millilitre per minute	$mL.min^{-1}$
Millilitres per kilogram body mass per minute	$mL.kg^{-1}$ body $mass.min^{-1}$
Millimoles per litre	mM
Millisecond	ms
Minute	min
Monocarboxylate transporters	MCT
Multi tasking test	MTT
Oxygen consumption	$\dot{V}O_2$
Oxygen pulse	O_2 pulse
Peak power output	W_{max}
Peroxisome proliferator-activated receptor gamma coactivator 1-alpha	PGC-1 α
Post-exercise ketosis	PEK
Placebo condition	PLA
Placebo plus carbohydrate condition	CHO+PLA
Pyruvate dehydrogenase	PDH
Rapid visual information processing task	RVP
Rating of perceived exertion	RPE
Reaction time task	RTI
Respiratory exchange ratio	RER
Respiratory minute volume	VE
Respiratory quotient	RQ
Revolutions per minute	RPM
R,S-1,3-butanediol acetoacetate diester	KDE
(R)-3-hydroxybutyl *(R)*-3-hydroxybutyrate ketone monoester	KME
Second	s
Solute ligand carrier	SLC
Solute ligand carrier protein 16A	SLC-16A
Standard deviation	SD
Succinyl-CoA:3-oxoacid CoA transferase	OXCT
Time trial	TT
Watt	W
Watts per second	$W.s^{-1}$
Yoyo-intermittent recovery test level 1	YoYo-IR1

Chapter 1

Introduction

Ketone bodies (KB), namely β-hydroxybutyrate (βHB), acetoacetate (AcAc) and acetone, are produced in the liver during physiological states and nutritional manipulations that result in reduced carbohydrate availability, including fasting, starvation and ketogenic diets (Robinson & Williamson, 1980; Laffel, 1999). The practical relevance for athletic seeking performance gains generated from these states are negligible (Burke, 2015). This led to the exploration of exogenous ketone supplements as means to achieve acute nutritional ketosis to raise ketone body concentrations without reducing carbohydrate availability. The use of exogenous ketone supplements in professional sport was rumoured as long ago as 2012, while their use was more recently confirmed in professional cycling (Abraham, 2015; Pinckaers et al., 2016). Since then several original research articles have been published investigating the effect of various exogenous ketone supplements on the metabolic response to exercise and physical and cognitive performance (Cox et al. 2016; Leckey et al. 2017; O'Malley et al. 2017; Rodger et al. 2017; Waldman et al. 2017; Holdsworth et al. 2017; Vandoorne et al. 2017). While interest has peaked only in the last few years, the work on ketones provided exogenously, either through injections or infusions goes back to the 1940s where their potential to alter whole body and local tissue metabolism was first realised. These data remained largely isolated until recently. The purpose of this introductory chapter is to explore the historical development of exogenous ketone supplements, and the current interest in these supplements for athletic performance. Chapter 2 will provide an extensive review of the physiology and metabolism of ketone bodies at rest, during exercise and in response to training.

History and development of exogenous ketone supplements

During the 1940s, a series of experiments identified βHB and AcAc as two of sixteen metabolites with the ability to increase the metabolic efficiency of animal sperm (Lardy & Phillips, 1945; Lardy et al., 1945). Simultaneously, βHB and AcAc injections were administered to study their effects on glucose metabolism in animal models (Nath & Brahmachari, 1944; Nath & Brahmachari 1946; Nath & Brahmachari, 1948; Tidwell & Axelrod, 1948; Tidwell & Nagler, 1952; Chari & Wertheimer, 1953). The blood glucose response to KB administration was divergent in these works. In a set of experiments administering βHB and AcAc injections to rabbits and guinea pigs, progressive hyperglycaemia was observed and it was hypothesised that KB cause a hypersecretion of insulin and damage of the pancreatic islet cells (Nath & Brahmachari, 1944; Nath & Brahmachari, 1946; Nath & Brahmachari, 1948). Conversely, hypoglycaemia was observed in rats following AcAc administration and glucose tolerance was unaltered following simultaneous AcAc/glucose administration. A "sugar sparing action of ketone bodies" was suggested, associated with decreased glycogenolysis and lower blood glucose concentrations (Tidwell & Axelrod, 1948; Tidwell & Nagler, 1952). Furthermore, KB inhibit glycolysis and increase the conversion of glucose to glycogen as demonstrated in rat heart skeletal muscle in vitro (Maizels et al., 1977), perfused heart model in dogs (Laughlin et al., 1994), and reduce hepatic glucose output when combined with somatostatin in type 2 diabetic patients (Henry et al., 1990).

The describing of KB for exogenous delivery in the novel form of ketone esters occurred some 40 years ago, and outlined a speculated primary role in parenteral nutrition (Brunengraber, 1997). The use of KB, whether as esters of AcAc or βHB, as a source of parenteral nutrition was devised as an alternative to carbohydrate to deliver more energy at a lower osmolality, avoid the pathophysiological side effects that accompany intravenous

glucose infusion, and avoid the catabolism of protein that accompanies instances of critical illness and trauma in humans (Birkhahn, 1983). Initially, glycerol monobutyrate was used due to its structural similarity to βHB with no toxic effects being observed in rats during infusion (Birkhahn et al., 1977). Monoacetoacetin, a monoester of glycerol and AcAc was also a viable intravenous substrate for rats. Body weight gains were observed and nitrogen balance in growing mice was maintained with 71% of energy needs met with monoacetoacetin infused over 7 d compared to isocaloric carbohydrate infusion, with total KB concentrations elevated to 0.4-1.2 mM in various animal models (Birkhahn et al., 1979; Birkhahn, 1983). To further increase total KB concentrations and the amount of energy per osmol provided parenterally, triesters of glycerol and acetoacetate were developed but were not completely water-soluble and therefore unsuitable for intravenous infusion (Brunengraber, 1997). The solution to this was to use βHB, improving the water-solubility of the compounds. Glycerol βHB mono- and triesters maintained nitrogen balance and body weight similar to that of isocaloric carbohydrate infusion when providing 50% of energy needs in rats over 7 d (Birkhahn et al., 1997). As well as glycerol, 1,3-butanediol (BD) can be bound via an esterification reaction to βHB or AcAc. BD is converted to D-βHB in the liver via of alcohol and aldehyde dehydrogenase (Kies et al., 1973), elevating circulating βHB concentrations and having metabolic effects such as protein sparing in burned rats (Maiz et al., 1985).

These data on the metabolic action of ketone esters remained largely isolated until the last 5 years, but KB infusion studies in humans from the late 1960s to 1980s contribute significantly to knowledge on the role of KB during exercise (Hagenfeldt & Wahren, 1968; Hagenfeldt & Wahren, 1971; Fery et al., 1974; Sherwin et al., 1975; Balasse et al., 1978; Fery & Balasse, 1983; Fery & Balasse, 1986; Fery & Balasse, 1988). KB disposal into skeletal muscle is elevated as much as fivefold during exercise; reflected by a decrease in circulating KB concentrations at the onset of exercise. βHB is the primary KB extracted from

circulation and a net production of AcAc is observed. This drop in βHB is accompanied by an increase in the metabolic clearance rate (MCR) and oxidation of KB in skeletal muscle (Fery & Balasse, 1986). MCR is a measure of the ability of tissues to remove ketones form the blood, analogous to arteriovenous difference per unit time, but when measured during exercise is taken to represent an index of the ability of exercise to stimulate the capacity of working muscle to extract and utilise ketones (Fery & Balasse, 1983; Balasse & Fery, 1989). Taken together, the earlier work on KB and their potential role in parenteral nutrition informed the development and safety of ketone esters, whereas the infusion work in humans undertaking exercise informed the role that KB may play as an alternative fuel source for skeletal muscle (Chapter 2).

Methods of exogenous ketone supplementation producing acute nutritional ketosis

Investigating effects of ketosis on skeletal muscle metabolism has been typically achieved by endogenous ketosis using fasting of various durations (Balasse & Fery, 1989), or by exogenous ketosis produced by either ketone salt ingestion (Johnson & Walton, 1972), or infusion of AcAc or βHB (Fery & Balasse, 1988; Mikkelsen et al., 2015). Endogenous ketosis may also be achieved by carbohydrate (CHO) restriction, particularly by ketogenic diets (Paoli et al., 2013). The practical relevance for athletes seeking performance gains of metabolic responses generated from prolonged fasting is negligible, whereas benefits of ketogenic dieting for performance with a high intensity component are equivocal (Burke, 2015). This has led to the exploration of exogenous ketone ingestion as a means to achieve acute nutritional ketosis. Importantly, because endogenous ketosis results in concomitant elevations in free fatty acids (FFA) and alterations in glucose, insulin and counter-regulatory hormones, isolating the metabolic effects specific to KB has proved challenging. Therefore,

exogenous ketone supplementation is a means to address these questions and explore potential for performance and therapeutic benefits.

Oral administration of KB in their free acid form is expensive and ineffective at producing ketosis, so buffering the free acid form with sodium/potassium/calcium salts has been explored and are commercially available. These too are relatively ineffective at increasing βHB concentrations, but may be improved by co-ingestion with medium chain triglycerides (C:8, C:10), at least in rats (Kesl et al., 2016). However, ingestion of large quantities of ketone salts (KS) is impractical due to resulting gastrointestinal distress, and potentially undesirable consequences of cation overload or acidosis (Veech, 2004).

The development of ketone esters provides an alternative method to increase βHB concentrations, and are well-tolerated in rodents and humans (Clarke et al., 2012; Cox et al., 2016; Kesl et al., 2016). Two prominent ketone esters in the published literature are the R,S-1,3-butanediol acetoacetate diester (KDE) (Kesl et al., 2016; Leckey et al., 2017), originally developed by Dr. Henri Brunengraber (Case Western Reserve University) as a metabolic therapy for parenteral and enteral nutrition (Desrochers et al., 1995), and the (R)-3-hydroxybutyl (R)-3-hydroxybutyrate ketone monoester (KME) (Clarke et al., 2012; Cox et al., 2016), originally developed by Dr. Richard Veech (National Institutes for Health) and Dr. Kieran Clarke (University of Oxford) to improve the physical and cognitive performance in warfighters (Ford and Glymour, 2014).

Acute ingestion of either ester can result in short-term (~0.5 to 6 h) nutritional ketosis indicated by βHB concentrations >0.5 mM (Clarke et al., 2012; Leckey et al., 2017). For KME, ingestion at a dose 573 $mg.kg^{-1}$ body mass (BM) resulted in βHB concentrations of ~3.0 mM after 10 minutes and rising to ~6.0 mM 30 min after ingestion (Cox et al., 2016). Ingestion of two 250 $mg.kg^{-1}$ body mass KDE at 50 and 30 min prior to a 31.2 km cycling time trial, respectively, elevates serum βHB concentrations to ~0.4 mM. Whole blood βHB

concentrations reached ~0.6 mM during the time trial and peaked at ~1.4 mM during a 1 h recovery period (Leckey et al., 2017). Acute nutritional ketosis is therefore achieved without the impracticality of prolonged fasting or ketogenic dieting.

Endogenous versus exogenous ketosis for athletic performance

The link between increasing muscle glycogen stores and enhanced exercise capacity is well-established, accomplished by increasing intake of dietary carbohydrates in the days prior to exercise or through exogenous carbohydrate feeding during exercise (Bergström et al., 1967; Coyle et al., 1986; Jeukendrup, 2004). Accordingly, sports nutrition guidelines recommend high carbohydrate intakes in preparation for and during competition to maximise muscle glycogen stores and improve performance (Burke et al., 2004). Despite this, low carbohydrate, high fat ketogenic diets are recommended in some quarters as a means to take advantage of the body's large fat reserves, namely adipose tissue and intramuscular triglycerides (IMTG), as a source of fuel during exercise (Volek et al., 2015). A period of "keto-adaptation" is often cited as necessary gain full benefit of a high fat diet (LCHF) for athletic performance. LCHF (<50g carbohydrate.day^{-1}) is associated with a preservation of submaximal exercise capacity (~60% $\dot{V}O2_{peak}$), a reduction in RER reflecting an increasing in fat utilisation during exercise and an elevation of circulating βHB to ~0.5-2.5 mM, depending on the length of the LCHF (Phinney et al., 1980; Phinney et al., 1983; Volek et al., 2016; McSwiney et al., 2018). Furthermore, muscle glycogen stores are maintained during 20 months of LCHF (~70% fat) despite elevated βHB concentrations, possibly explaining the preservation of exercise capacity and the ability to synthesise muscle glycogen from gluconeogenic substrates (Volek et al., 2016). These dietary approaches can be considered as "endogenous ketosis", but are proposed to impair high intensity exercise performance through an attenuation of pyruvate dehydrogenase activity (PDH), a key glycolytic enzyme

(Burke, 2015). This performance impairment was demonstrated as a reduction in 1 km sprint power output following 6 d adaptation to a LCHF (~68% fat), while 100 km time trial performance was maintained (Stellingwerff et al., 2006; Havemann et al., 2006). Of note is the brief (<1 wk) adaptation period to the LCHF, which proponents of the diet say is too short to fully adapt to endogenous ketosis as an alternative fuelling strategy (Volek et al., 2015). However, while 3 wk adaptation to a LCHF improves maximal aerobic capacity ($\dot{V}O2_{peak}$) in a group of Australian elite race walkers, it conversely impairs gains in performance in response to a 3 wk intense training camp in elite race walkers (Burke et al., 2017).

"Exogenous ketosis", also known as the aforementioned acute nutritional ketosis, can be produced through ingestion of a ketone ester or ketone salt (Clarke et al., 2012; Cox et al., 2016; Stubbs et al., 2017) but does not require a LCHF to elevate βHB concentrations, and results in a similar increase in βHB concentrations to endogenous ketosis. Elevations in βHB concentrations are achieved with concomitant exogenous ketone supplement and carbohydrate intake, regardless of prior feeding status (Stubbs et al., 2017; Myette-Côté et al., 2018). Exogenous ketone ingestion aims to circumvent the impairment of high intensity exercise performance in athletes by elevating βHB concentrations without the impractical but necessary low carbohydrate intake implemented during endogenous ketosis. However, similarly to endogenous ketosis, rats supplemented for 5 d with exogenous ketones exhibit a reduction in cardiac PDH activity versus rats fed a high carbohydrate diet (Murray et al., 2016), but this inhibition remains to be directly confirmed either in human skeletal muscle, or in response to a single bolus ingestion. Nevertheless, given the effects of acute ingestion of KME to reduce glycolytic flux during exercise (Cox et al., 2016), it is likely that some of these effects are being mediated via inhibition of PDH.

Ketogenic supplements

Ketones esters and KS are exogenous ketone supplements orally ingested with the aim of elevating circulating βHB concentrations. βHB has two enantiomers, designated D- and L-, or R- and S-, respectively. Currently-available commercial βHB assays and handheld point-of-care monitors determine the concentration of the D- enantiomer. D-βHB is the circulating and primary form of βHB (Tsai et al., 2006), and L-βHB is not a substrate for mitochondrial 3-hydroxybutyrate dehydrogenase, a key enzyme in the ketolytic pathway, and thus is not metabolised to AcAc (Scofield et al., 1982). The constituent parts of ketone esters and KS are different and this alters the metabolic response to the respective ketogenic supplements after acute ingestion. Ketone esters, namely KME and KDE, are D-βHB and AcAc molecules respectively, attached via an ester bond either to another ketone body or a ketone body precursor such as BD. KS are a racemic mixture of D/L-βHB or non-racemic D-βHB molecules attached to a sodium/calcium/potassium mineral salt (Figure 1.1). All investigations in to the efficacy of elevating βHB concentrations using KS have used commercially-available supplements containing a racemic mixture of βHB, typically containing 50% of the D- and L-enantiomers (Stubbs et al., 2017; O'Malley et al., 2017; Rodger et al., 2017; Fischer et al., 2018; Waldman et al., 2018). KME has a chiralic purity of 99%, meaning it exclusively contains the D-βHB isomer, whereas commercially-available KS have a purity of 50%, making them less effective at elevating the metabolically-active D-βHB enantiomer (Stubbs et al., 2017). One serving of KME (DeltaG, HVMN Ketone, HVMN, United States) provides 25g D-βHB, whereas one serving of KS (KetoCana, KetoSports, United States) provides 11.7 g βHB comprising of 50% each of the D- and L-βHB enantiomers.

In addition to the ingestion of KB-containing formulations, medium chain

triglycerides are ketogenic agents and may be coingested with KS to augment the βHB response to KS ingestion (Kesl et al., 2016). Briefly, medium chain triglycerides are fatty acids 6 to 12 carbons in length and are transported to the liver directly via the portal vein and oxidised rapidly (Bach & Babayan, 1982). Acute ingestion of 25 g medium chain triglyceride prior to exercise elevates βHB to ~0.5 mM, lowering blood glucose concentrations but has no effect on glycogen utilisation during 60 min cycling at 60% $\dot{V}O2_{peak}$ (Decombaz et al., 1983). Simultaneous carbohydrate and medium chain triglyceride ingestion increases the rate of medium chain triglyceride oxidation compared to medium chain triglyceride ingestion alone (Jeukendrup et al., 1995), with the latter impairing performance in a cycling time trial due to a high incidence of gastrointestinal distress (Jeukendrup et al., 1998). However, this impairment in performance was not observed with concomitant carbohydrate and medium chain triglyceride ingestion (Jeukendrup et al., 1998).

The original article on the effect of a (R)-3-hydroxybutyl (R)-3-hydroxybutyrate ketone monoester (KME) on cycling time trial performance remains the *only* report of a performance benefit of an exogenous ketone supplement (Cox et al., 2016). Ingestion of 573 $mg.kg^{-1}$ body mass of KME improved 30 min maximal distance time trial performance by ~2%, whereas 31.2 km cycling time trial performance was impaired by ~2% with ingestion of the R,S-1,3-butanediol acetoacetate diester (Leckey et al., 2017). Racemic ketone salts have no performance benefit for short duration, high intensity efforts (O'Malley et al., 2017; Rodger et al., 2017; Waldman et al., 2018). At the time of commencing our experimental work, the *Cell Metabolism* report (Cox et al., 2016) was the only published work in the area and informed the dosing and timing strategies employed in studies 1, 2 and 3 of this thesis.

Given the increasing interest but relative lack of scientific research on exogenous ketone supplements, there is a need to investigate whether ketogenic supplements alter the

metabolic response to various exercise challenges and improve athletic performance. With these gaps in knowledge in mind, three human intervention studies were undertaken to investigate whether administration of βHB in the form of KS or ketone esters altered the metabolic response to exercise and/or improved physical and cognitive performance in response to various exercise challenges in trained athletes.

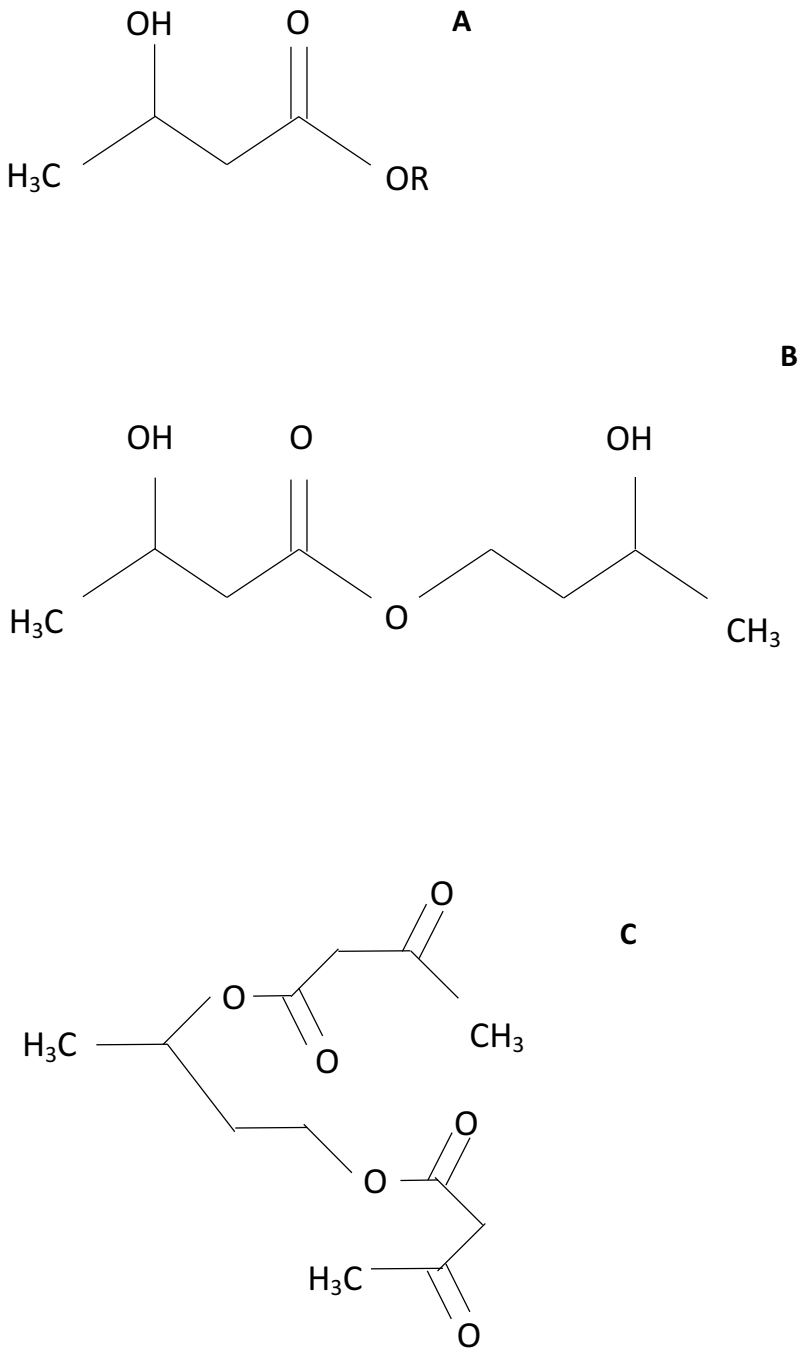

Figure 1. 1 Chemical structures of ketogenic supplements

A, racemic ketone salts. B, the (R)-3-hydroxybutyl (R)-3-hydroxybutyrate ketone monoester. C, R,S-1,3-butanediol acetoacetate diester.

Aims of the Thesis

- During pilot testing, discover the optimal dose and timing of racemic ketone ingestion to maximise peak βHB concentrations after acute ingestion of racemic ketone salts.
- Use this dose and timing information to investigate the effect of racemic ketone salts on the metabolic response to a graded exercise session in trained cyclists. Specifically, are the βHB concentrations achieved similar to those of the (R)-3-hydroxybutyl (R)-3-hydroxybutyrate ketone monoester and will they cause the characteristic reduction in plasma glucose and attenuate the exercise induced rise in plasma lactate concentrations.
- Investigate whether carbohydrate plus the (R)-3-hydroxybutyl (R)-3-hydroxybutyrate ketone monoester has an ergogenic effect on short duration, high intensity exercise performance during a 75 min soccer simulation protocol and a subsequent intermittent run to volitional exhaustion in team sport athletes compared to carbohydrate ingestion alone. It is of interest to whether the metabolic effects of (R)-3-hydroxybutyl (R)-3-hydroxybutyrate ketone monoester ingestion, namely a reduction in plasma glucose and an attenuation in the exercise-induced rise in plasma lactate, are observed in this intermittent sporting context.
- Investigate whether ingestion of carbohydrate plus the (R)-3-hydroxybutyl (R)-3-hydroxybutyrate ketone monoester has an ergogenic effect on 10 km self-paced, treadmill based time trial performance following a 1 h pre load at 65% $\dot{V}O2_{peak}$ compared to carbohydrate alone.
- Investigate whether ingestion of carbohydrate plus the (R)-3-hydroxybutyl (R)-3-hydroxybutyrate ketone monoester improves cognitive performance in intermittent

and endurance contexts compared to carbohydrate ingestion alone. Reaction time, decision-making and sustained attention will be measured.

Chapter 2

Sections from this literature review are included in the published Journal of Physiology article (Appendix A): **Evans M**, Cogan KE, Egan B (2017) Metabolism of ketone bodies during exercise and training: physiological basis for exogenous supplementation. *Journal of Physiology* 595(9):2857–71.

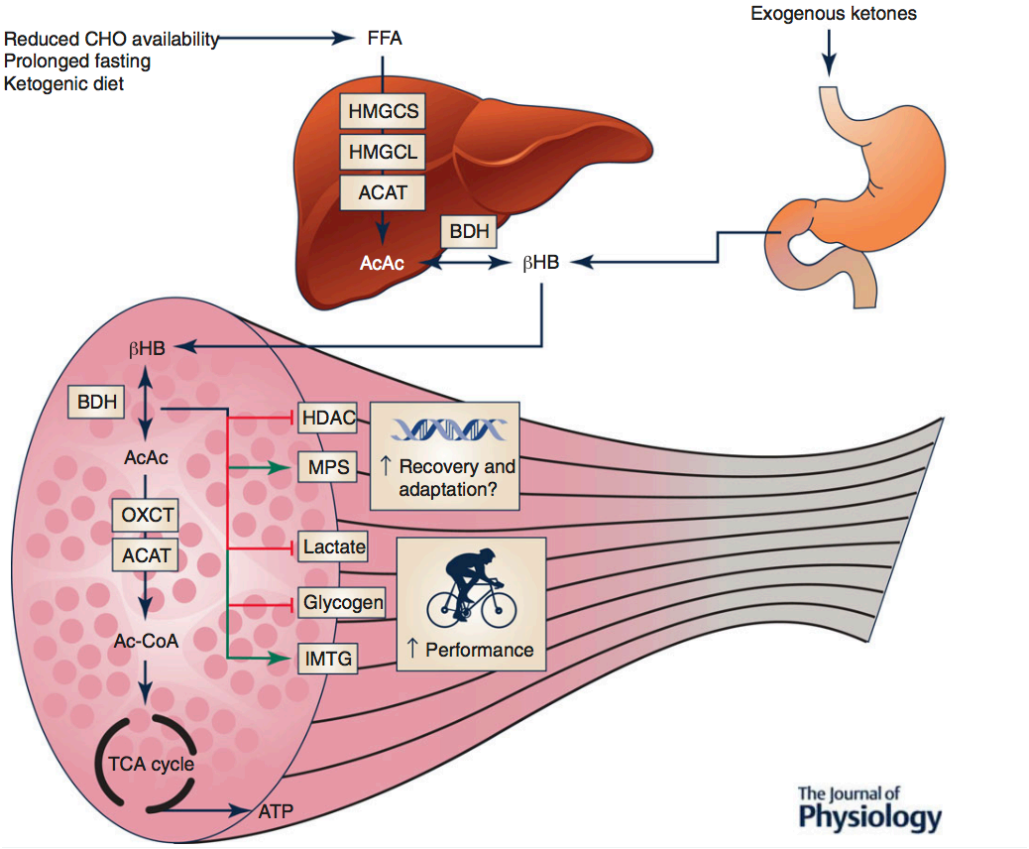

Figure 2.1 Overview of ketogenesis, ketolysis and the metabolic and ergogenic potential of exogenous ketones.
Endogenous ketogenesis and exogenous ketone ingestion result in an increase in circulating βHB. βHB enters the muscle where it alters the metabolic response to exercise and may result in improved recovery and adaptation from exercise training or improved physical performance.

Introduction

Over the past century, exercise physiologists have appreciated the role of carbohydrate and fat in energy provision to exercising skeletal muscle. Much of the work examining the metabolic response during exercise and the impact of exercise on metabolic regulation and adaptive responses to training has focussed on the relative contribution of

these fuels (Egan & Zierath, 2013). Optimising training and nutrition strategies by manipulating the relative intakes of these macronutrients is central to supporting elite sports performance (Cermak & van Loon, 2013; Bartlett et al., 2015; Burke, 2015). An alternative fuel source to CHO and fat are ketone bodies (KB), namely acetoacetate (AcAc), acetone, and β-hydroxybutyrate (βHB), which are produced in the liver during physiological states and nutritional manipulations that result in reduced CHO availability, most commonly during prolonged fasting, starvation, and ketogenic [very low CHO (~5%), low protein (~15%), high fat (~80%)] diets (Robinson & Williamson, 1980; Laffel, 1999). This relative glucose deprivation and concomitant elevation in circulating free-fatty acids results in the production of KB to replace glucose as the primary fuel for peripheral tissues such as the brain, heart and skeletal muscle in these states.

Aside from a role as an alternative fuel source, KB exert a range of metabolic effects including attenuating glucose utilisation in peripheral tissues, anti-lipolytic effects on adipose tissue, and potential attenuation of proteolysis in skeletal muscle (Figure 2.1) (Robinson & Williamson, 1980). KB are utilised by working muscle during exercise (Fery & Balasse, 1986; Fery & Balasse, 1988), and the capacity to uptake and oxidise KB during exercise is higher in exercise-trained skeletal muscle (Winder et al., 1975). Despite these observations, in addition to a glucose sparing action (Maizels et al., 1977) and potential to lower the exercise-induced rise in plasma lactate concentrations (Fery & Balasse, 1988), the potential performance benefits of KB when provided as an exogenous fuel source had until recently received little attention, but had been postulated (Cox & Clarke, 2014; Pinckaers et al., 2016). Since then several articles have been published investigating the effect of various exogenous ketone supplements on the metabolic response to exercise and physical and cognitive performance (Cox et al. 2016; Leckey et al. 2017; O'Malley et al. 2017; Rodger et al. 2017; Holdsworth et al. 2017; Vandoorne et al. 2017; Waldman et al., 2018). Apart from a role as

an alternative fuel source, KB may act as signalling molecules to regulate gene expression and adaptive responses (Shimazu et al., 2013; Zou et al., 2016). Moreover, therapeutic roles for KB have long been proposed in a variety of disease states including aberrant glucose metabolism, genetic myopathies, hypoxic states and neurodegenerative pathologies (Veech, 2004). For therapeutic effects, exogenous ketones are ingested in the form of βHB salts or ketone esters to produce acute (~0.5 to 6 h) nutritional ketosis (Clarke et al., 2012; Kesl et al., 2016), but a surge in interest in KB as a performance aid for athletes arose when ketone ester supplementation was confirmed in professional cycling (Abraham, 2015; Pinckaers et al., 2016).

Exercise metabolism and biochemistry

Overview

Skeletal muscle has the capacity to alter the rate of energy production in response to movement, powered by actin-myosin crossbridge cycling contraction (Podolsky and Schoenberg, 1983). Exercise causes skeletal muscle to rapidly increase its rate of ATP hydrolysis, providing an immediate source of energy for muscular work by increasing ATP turnover more than 100-fold compared to resting values (Gaitanos et al., 1993). Metabolic pathways that drive ATP production are activated during exercise that help to maintain ATP stores, facilitating the continuation of muscular work. Skeletal muscle is the primary site of carbohydrate and lipid metabolism for energy production, illustrated by a greater than 30-fold demand increase in intramuscular oxygen consumption during strenuous exercise and an approximate 70- to 100- fold increase in tricarboxylic acid cycle flux under similar conditions (Anderson and Saltin, 1985; Gibala et al., 1998). The relative contribution of CHO and lipid to total energy provision during exercise is dependent on the modality, the frequency, intensity, and duration of individual exercise sessions (Egan and Zierath, 2013).

Substrate utilisation during exercise

The relative contribution of different metabolic pathways to energy provision is dependent on the relative intensity and power output of the exercise bout. Power output determines the ATP demand while intensity determines the relative contributions of CHO, lipid and other fuel sources. Isotope tracer methodology has provided us with a detailed view of the impact of these factors on substrate utilisation during exercise (Romijn et al., 1993; van Loon et al., 2001). At low to moderate intensities of exercise (<65% $\dot{V}O_{2peak}$), the primary source of fuel for skeletal muscle is glucose, derived from hepatic glycogenolysis, and free fatty acids, liberated from adipose tissue. These responses are dependent on various hormonal responses, including adrenaline, noradrenaline, insulin, and glucagon) (Kjaer, 2006). As exercise intensity increase (>65 $\dot{V}O_{2peak}$), the contribution of CHO increases while the use of FFA declines. This trend continues up to maximal intensities, where the vast majority of energy is being provided by muscle glycogen (Van Loon et al., 2001). Conversely to exercise intensity, as exercise duration increase (> 60 min) lipid oxidation increases (Romijn et al., 1993) (Figure 2.2). Muscle glycogen, the predominant fuel during moderate to intense exercise, is converted to glucose in a process known as glycogenolysis. This process is under the control of the enzyme glycogen phosphorylase, regulated by allosteric modulation by AMP and IMP (Hargreaves, 2006). The end of product of glycogenolysis is glucose molecules that are converted to glucose-6-phosphate and subsequently pyruvate in the cytoplasm before crossing the mitochondrial membrane, converted to acetyl Co-A and incorporated in to the tricarboxylic acid cycle to produce ATP. As the duration of exercise increases, or the intensity of exercise decreases, the contribution of lipids to fuel provision increases. FFA, liberated from adipose tissue and intramuscular trigycerides are the two sources of lipids that muscle can oxidise during exercise. Lipids, in the form of triglycerides, are stored in adipose tissue and sequentially broken down to glycerol and three fatty acids by

the enzymes adipose triglyceride lipase, hormone sensitive lipase and monoacylglycerol lipase under the control of the hormone insulin and the catecholamines. Each fatty acid passes out of the adipocyte in to the blood and are transported across the plasma membrane of the muscle cell via fatty acid transports, including fatty acid binding protein, fatty acid transport protein and fatty acid translocase. Fatty acids are transported across the carnitine shuttle and undergo β-oxidation in the mitochondria in the mitochondria to produce acetyl CoA, which enters the tricarboxylic acid cycle to produce ATP and fuel muscular work (Figure 2.3).

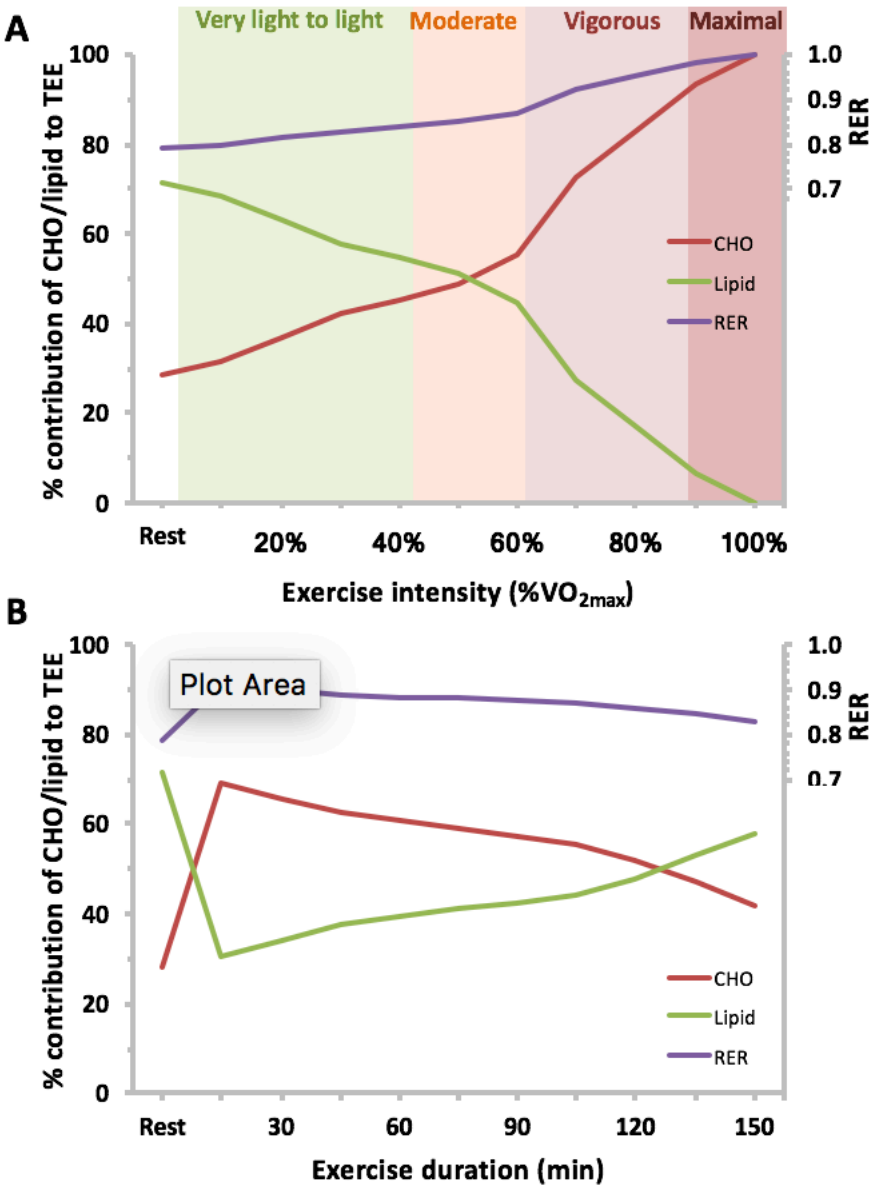

Figure 2. 2 The effect of exercise intensity and exercise duration on substrate metabolism during acute exercise

(A) Substrate contribution to exercise of increasing intensity. Up to 30%VO2max, oxidation of lipid sources (mostly plasma FFAs) accounts for themajority of energy provision. As exercise intensity increases, absolute CHOoxidation rate and relative contribution to energy provision increases. The lipidoxidation rate increases up to60%–70% VO2max, after which it declines asintensity increases. In relative terms, the contribution of lipid oxidation toenergy provision decreases proportionally with increasing exercise intensity,as reflected by a steady rise in RER.(B) Substrate contribution to exercise at a fixed intensity (e.g., 65%VO2max) foran extended duration. An initial rise in RER occurs at the onset of exercise,reflecting the increase in relative contribution of CHO to energy provisioncompared to resting metabolism. Thereafter, a small but steady decline in RERis observed with extended duration of exercise, reflecting the declining relativecontribution of CHO to energy provision as a function of the increasing relativecontribution of lipid.

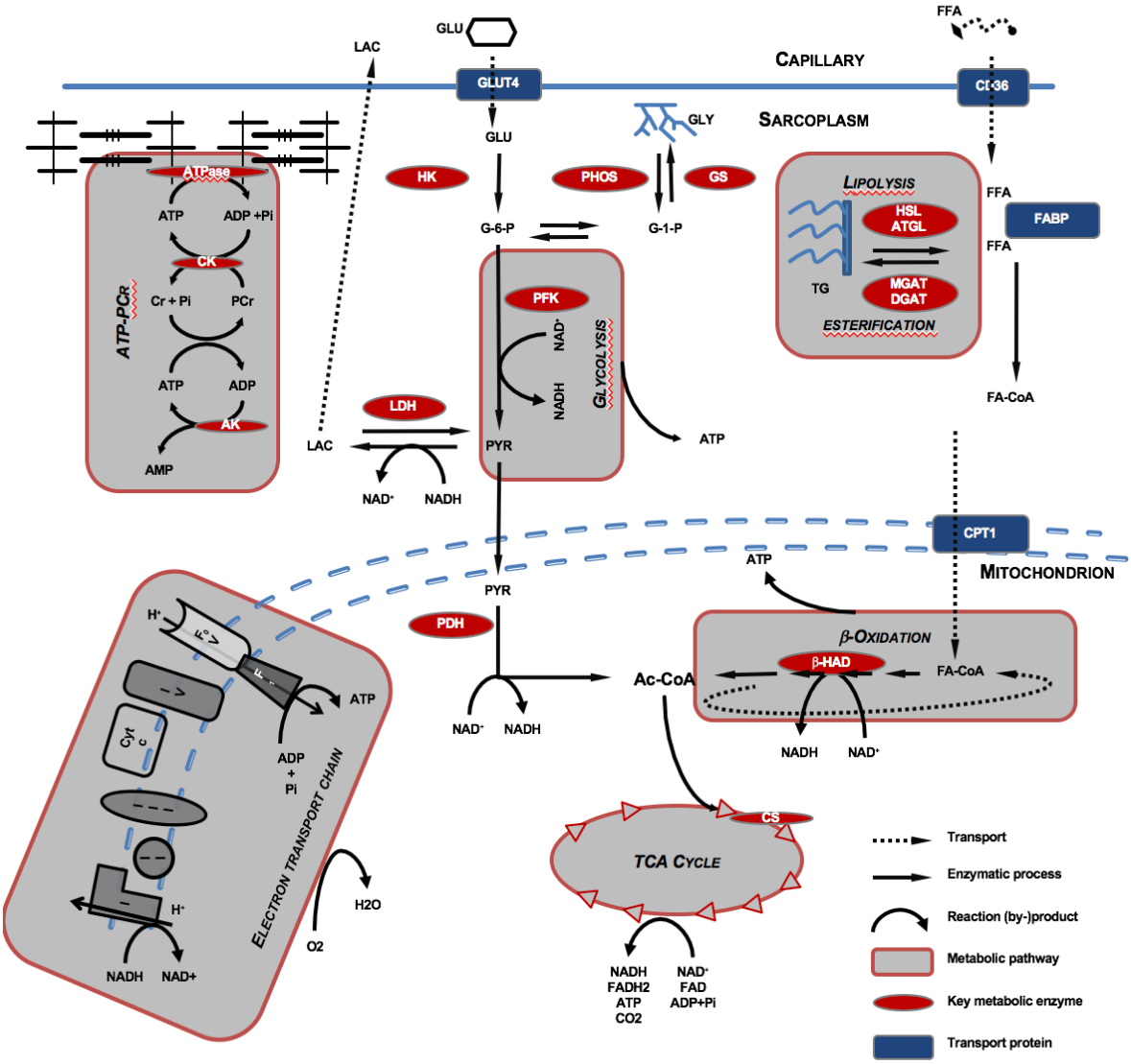

Figure 2. 3 Energy provision in skeletal muscle during exercise

ATP hydrolysis, catalyzed by myosin ATPase, powers skeletal muscle contraction. Metabolic pathways of ATP generation in skeletal muscle include (1) the ATP-phosphagen system wherein the degradation of PCr by creatine kinase (CK) produces free Cr and Pi, which is transferred to ADP to re-form ATP; the adenylatekinase (AK) (myokinase) reaction catalyzes the formation of ATP and AMP from two ADP molecules; (2) anaerobic glycolysis, where glucose-6-phosphate derivedfrom muscle glycogen (GLY) (catalyzed by glycogen phosphorylase, PHOS) or circulating blood glucose (GLU) (catalyzed by hexokinase, HK), is catabolized

topyruvate (PYR), which is reduced to lactate (LAC) by lactate dehydrogenase (LDH), and produces ATP by substrate level phosphorylation; (3) processesofcarbohydrate (glycolysis) and lipid (b-oxidation) metabolism producing acetyl-CoA (Ac-CoA), which enters the tricarboxylic acid (TCA) cycle in the mitochondria,coupled to oxidative phosphorylation in the electron transport chain (ETC). The two main metabolic pathways, i.e., glycolysis and oxidative phosphorylation, arelinked by the enzyme complex pyruvate dehydrogenase (PDH). GLUT4 facilitates glucose uptake to the sarcoplasm, which may undergo glycolysis or during rest/inactivity, be stored as glycogen via glycogen synthase (GS). Fatty acyl translocase (FAT/CD36) facilitates long-chain fatty acid transport at the sarcolemma, and,in concert with fatty acid binding protein (FABPpm) and carnitine palmitoyltransferase 1 (CPT1), across the mitochondrial membrane. FFAs entering the cell maybe oxidized viab-oxidation or be diverted for storage as IMTG via esterification by monoacylglycerol acyltransferase (MGAT) and diacylglycerol acyltransferase(DGAT). Liberation of FFAs from IMTG stores via lipolysis in skeletal muscle during exercise occurs via the activities of HSL and ATGL. All pathways of ATPgeneration are active during exercise, but the relative contribution of each is determined by the intensity and duration of contraction, as a function of the relativepower (rate of ATP production) and capacity (potential amount of ATP produced). CS, citrate synthase; Cyt c, cytochromec; PFK, phosphofructokinase

Overview of ketone body metabolism

Ketone bodies in circulation

Plasma KB concentrations reflects the balance between hepatic production ("ketogenesis") and peripheral breakdown and utilisation ("ketolysis") in extra-hepatic tissues, both of which are under various levels of control as detailed in previous reviews (Robinson & Williamson, 1980; Laffel, 1999). Ketogenesis is an evolutionarily-conserved adaptive response playing a critical role in survival during an energy crisis by providing a substrate for the brain, which cannot utilise FFA as a fuel source. AcAc, acetone, and βHB comprise the KB, although βHB is not technically a ketone because the ketone moiety has been reduced to a hydroxyl group. AcAc and βHB are short-chain, four carbon organic acids that act as FFA-derived circulating substrates to provide energy to extra-hepatic tissues, whereas the contribution of acetone, readily generated by the spontaneous decarboxylation of AcAc, to energy provision is negligible. Plasma KB concentrations are <0.1 mM in the postprandial state, whereas hyperketonemia is accepted as KB concentrations exceeding 0.2 mM (Robinson & Williamson, 1980). Various states of CHO restriction, depletion and dysregulation produce hyperketonemia to different degrees (Figure 2.4).

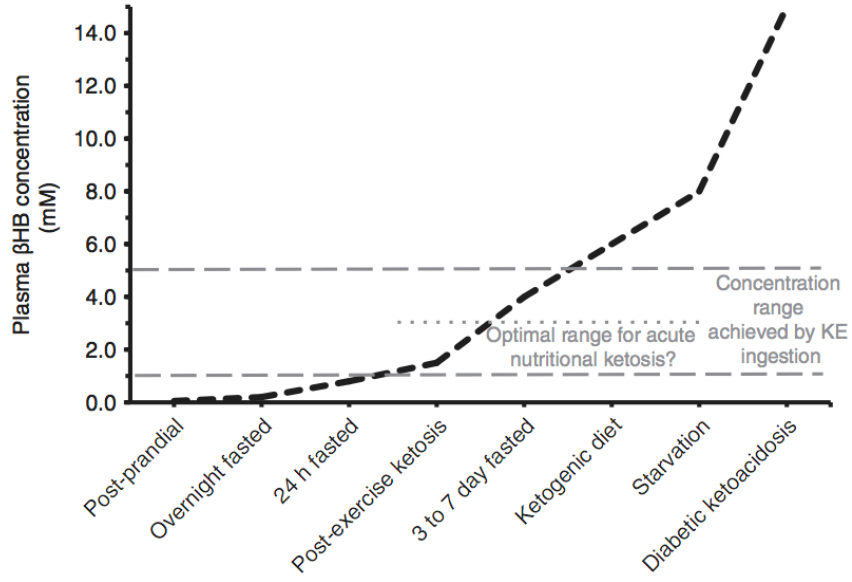

Figure 2.4 Changes in βHB concentrations under various physiological states.
Plasma [KB] is <0.1 mM in the postprandial state when consuming high CHO or high protein meals, and rises upward after an overnight fast, and with ketogenic dieting, prolonged fasting, starvation, and pathological states of ketoacidosis.

Ketogenesis

The primary substrate for ketogenesis are FFA liberated from adipose tissue. Ketogenic amino acids, namely leucine, lysine, phenylalanine, isoleucine, tryptophan, and tyrosine also serve ketogenesis, but are likely to contribute to less than 5% of circulating KB (Thomas et al., 1982). The rise in FFA is consequent to the stimulation of lipolysis as a result of declines in plasma glucose and insulin that are characteristic of reduced CHO availability. Factors stimulating ketogenesis include an elevated glucagon-to-insulin ratio and decline in hepatic glycogen concentration, while reduced blood flow to the liver or elevations in [KB] suppress ketogenesis (Robinson & Williamson, 1980; Laffel, 1999). Ketogenesis involves a series of sequential reactions beginning with acetyl CoA (Ac-CoA) and acetoacetyl CoA (AcAc-CoA), and ending with the liberation of AcAc (Figure 2.5). Some AcAc is exported, but the majority is reduced to βHB in an NAD^+/NADH-coupled near equilibrium reaction catalysed by 3-hydroxybutyrate dehydrogenase (BDH), in which the equilibrium constant

favours βHB formation. These KB are transported into the circulation via the solute ligand carrier (SLC) protein 16A (SLC16A) family of monocarboxylate transporters (MCTs) in mitochondrial and sarcolemmal membranes.

Ketolysis in extra-hepatic tissues

In peripheral tissues, KB, primarily in the form of βHB, enter the mitochondrial matrix again via MCT1-mediated transport. βHB is re-oxidised to AcAc via BDH after which sequential reactions result in the generation of two molecules of Ac-CoA (Figure 2.5). These are incorporated into the TCA cycle via citrate synthase for terminal oxidation and production of ATP, which in skeletal muscle contributes to fuelling muscular work (Fery & Balasse, 1986, 1988). Succinyl-CoA:3-oxoacid CoA transferase (OXCT) is essential for ketolysis in extra-hepatic tissues, with very low abundance in hepatocytes explaining the lack of ketolytic activity in these cells (Robinson & Williamson, 1980).

Activity of OXCT is highest in heart and kidney, followed by skeletal muscle and the brain (Robinson & Williamson, 1980), but because skeletal muscle accounts for ~40% of body mass in adult humans, this organ accounts for the highest fraction of total KB metabolism at rest (Balasse & Fery, 1989; Laffel, 1999). Beginning almost 50 years ago, models using various durations of fasting, and combined with primed constant infusion of radiolabelled either AcAc or βHB tracers and arteriovenous difference measures to quantify KB turnover, established that skeletal muscle is a major site of ketolysis at rest (Hagenfeldt & Wahren, 1968; Owen & Reichard, 1971; Wahren et al., 1984; Elia et al., 1990; Mikkelsen et al., 2015). Skeletal muscle has a high affinity to KB, but because of low circulating concentrations under normal conditions, the contribution to energy provision in muscle is less than 5%, and FFA are the main source of energy provision in the post-absorptive state. The relationship between ketone oxidation and KB concentrations is curvilinear such that

contribution to energy provision in skeletal muscle rises to ~10% after an overnight fast (Hagenfeldt & Wahren, 1968; Owen & Reichard, 1971), 20% to 50% after 72 h of fasting (Owen & Reichard, 1971; Elia et al., 1990), but declines to ~15% after 24 days of starvation (Owen & Reichard, 1971). Thus, skeletal muscle demonstrates saturation kinetics for the KB concentration-oxidation relationship, with saturation likely between 1 and 2 mM as demonstrated by fasting of various durations (compiled in (Balasse & Fery, 1989)) or step-wise βHB infusion (Mikkelsen et al., 2015).

Measurement of βHB

The majority of studies on acute exogenous ketone ingestion use handheld point-of-care meters with appropriate reagent strips to measure whole blood βHB concentrations (Precision Neo handheld monitor, Freestyle Optium handheld monitor, Precision Xtra handheld monitor Abbott Laboratories, Witney, UK; Glucomen Lx plus-meter, Menarini Diagnostics, Firenze, Italy) (O'Malley et al., 2017; Rodger et al., 2017; Stubbs et al., 2017; Myette-Côté et al., 2018; Stubbs et al., 2018; Waldman et al., 2018; Leckey et al., 2017). Ketone body reagent strips use a βHB dehydrogenase enzyme-based amperometric strip to establish whole blood βHB (Ceriotti et al., 2014). The use of handheld monitors is preferable to urinary ketone measurement due to the inability of nitroprusside, present in the urinary sticks, to react with βHB, the predominant ketone body in circulation (Guimont et al., 2015). Other studies have used laboratory methods, including reagent/colorimetric kits for measurement of plasma βHB (Randox, Daytona; Sigma Aldrich; ABX Pentra, France) (Cox et al., 2016; Leckey et al., 2017; Vandoorne et al., 2017). The Optium Freestyle handheld monitor (Abbott Laboratories, Berkshire, UK) overestimates βHB concentrations but falls within the coefficient of variation (10%) with a laboratory method (Guimont et al., 2015), but this overestimation increases with higher βHB concentrations (>5.0mM) (Ceriotti et al.,

2014). Confounding factors for βHB measurement with handheld monitors include high haematocrit and ascorbic acid concentrations, both of which cause overestimation of βHB with the Freestyle Optium monitors (Ceriotti et al., 2014; Dhatariya, 2016). Furthermore, βHB concentrations were overestimated by ~0.4-0.5 mM during exercise using a handheld monitors compared to direct assay-based laboratory methods (Leckey et al., 2017). These reports suggest elevations in βHB concentrations via exogenous ketone ingestion must be critically evaluated based on the measurement method and any confounding factors that could affect such methods.

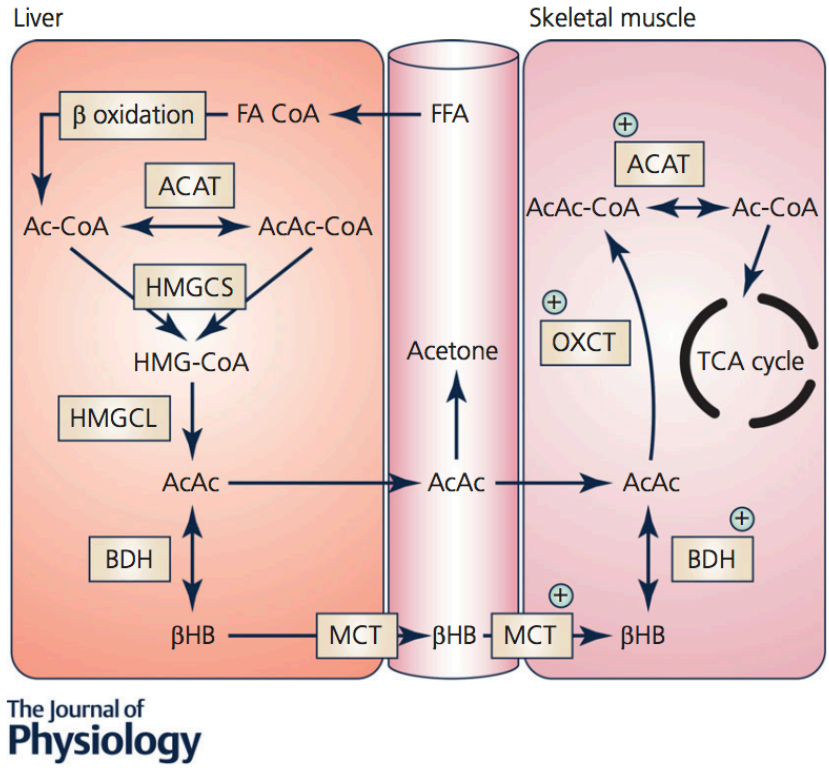

Figure 2.5 Metabolic pathways of ketone body metabolism in liver and skeletal muscle
During ketogenesis, FFA are converted to fatty acyl CoA, enter hepatic mitochondria via MCT transport and undergo β-oxidation to acetyl CoA. Sequential reactions lead to the reduction of the central ketone body, AcAc, to βHB. In the muscle βHB is reoxidised to AcAc. Two molecules of acetyl-CoA are formed during the process of ketolysis

Effect of aerobic exercise training on enzymes of ketogenesis and ketolysis

Adaptations to exercise training reduce perturbations to homeostasis during subsequent bouts of exercise, and thereby enhance resistance to fatigue. Central to these effects are enhanced respiratory capacity and contractile parameters, and importantly adaptations that contribute towards maximising delivery and utilisation of circulating substrates (Egan & Zierath, 2013). Therefore, if KB make a meaningful contribution to energy provision during exercise, it is pertinent to explore analogous regulation in skeletal muscle. Training-induced changes in expression and activities of enzymes of ketolysis in skeletal muscle have not been described in humans, but differences in KB metabolism during and after exercise between trained and untrained individuals have been reported (Johnson et al., 1969; Johnson & Walton, 1972; Rennie et al., 1974; Rennie & Johnson, 1974). The general pattern is for attenuation in trained individuals of the post-exercise rise in KB concentrations, but this is influenced by nutritional manipulation and relative exercise intensity, the latter of which has often been poorly controlled (see later sections).

Nevertheless, circulating concentrations reflect the balance between ketogenesis and ketolysis, these differences may be explained by the factors influencing one or both. For ketogenesis, data are limited but suggest that in exercise-trained rodents enzymatic activity of BDH or ACAT (Winder et al., 1974), or HMGCS (Askew et al., 1975) is unaltered in liver, and, in fact, the overall activity of the ketogenic pathway may be lower (El Midaoui et al., 2006) compared to untrained rodents. In these rodent models of intense aerobic exercise training, the activities of the ketolytic enzymes BDH, OXCT and ACAT are higher in trained skeletal muscle (Winder et al., 1974; Askew et al., 1975; Winder et al., 1975; Beattie & Winder, 1984). This coincides with two- to three-fold higher ex vivo rates of βHB and AcAc oxidation in gastrocnemius muscle homogenates presented with concentrations of both βHB and AcAc at 0.1 and 0.5 mM (Winder et al., 1973; Winder et al., 1975).

In terms of muscle fibre type, enzymatic activities of BDH, OXCT and ACAT are all highest in type I fibres, intermediate in type IIA fibres, and lowest in type IIB fibres of rats (Winder et al., 1974). BDH is essentially undetectable in type IIB muscle fibres, and across the fibre types BDH activity is much lower than activities of OXCT and ACAT (Winder et al., 1974). Although OXCT is essential for ketolysis, BDH activity is, therefore, potentially rate-limiting in skeletal muscle. When rats performed 12 weeks of treadmill running, compared to sedentary rats BDH activity was almost three-fold higher in type I fibres, but six-fold higher in type IIA fibres of trained skeletal muscle, resulting in concentrations comparable to the type I fibres (Winder et al., 1974). OXCT activity was 26% higher in type I, and approximately two-fold higher in type IIA and IIB fibres, whereas ACAT activity was 40% to 45% higher in all three fibre types in trained skeletal muscle (Winder et al., 1974). Similarly, in skeletal muscle from mice with 8 weeks of access to running wheels, the difference compared to sedentary mice was greater for BDH mRNA expression (~two-fold higher than sedentary) compared to differences in OXCT and ACAT mRNA expression (~30% to 50% higher) (Svensson et al., 2016). These changes in ketolytic enzymes are localised to the working muscle given the absence of change after training in the heart (Askew et al., 1975), kidney and brain (Winder et al., 1974).

In terms of KB transport into skeletal muscle, similarly to the ketolytic enzymes, MCT1 protein expression is highest in type I fibres, lowly expressed in type II fibres, and correlates well with muscle oxidative capacity (Bonen, 2001). Elevated MCT1 protein expression after exercise training is well-established for human skeletal muscle, and increases occur in an intensity-dependent manner (Thomas et al., 2012). Using a rodent perfused hindlimb model, the capacity for uptake of KB in skeletal muscle at 1 mM each of βHB and AcAc was higher in an aerobically-trained group of rats, with uptake of total KB, AcAc and βHB 33%, 27% and 53% higher, respectively, compared to untrained rats (Ohmori et al.,

1990). Similarly, βHB clearance during a βHB tolerance test is higher in mice given 8 weeks of running wheel access, or with enhanced oxidative capacity consequent to skeletal muscle overexpression of PGC-1α, a transcriptional co-activator and master regulator of mitochondrial biogenesis in adaptive responses such as exercise training (Svensson et al., 2016). In both conditions, this coincides with elevated expression of MCT1 and the ketolytic enzymes in skeletal muscle. Therefore, the uptake and utilisation of KB in skeletal muscle is likely to be greatest in those individuals that are highly trained with a high proportion of type I muscle fibres and a high oxidative capacity in skeletal muscle.

Ketone body metabolism during exercise

The existing literature on fuel selection during exercise has focused almost exclusively on utilisation of CHO and fat, but skeletal muscle has the ability to resynthesize ATP from other substrates including protein, lactate, and KB (Fery & Balasse, 1986; Mazzeo et al., 1986; Fery & Balasse, 1988; Wagenmakers et al., 1991). With increasing exercise intensity, the contribution of substrates to energy provisions shifts from blood-borne FFA and glucose to increased reliance on intramuscular fuel stores, namely intramuscular triglyceride and muscle glycogen, such that at moderate to high intensities (>75%VO_{peak}) of exercise, muscle glycogen is the main source of energy provision (van Loon et al., 2001). This pattern is readily altered by nutritional manipulation such as CHO loading and acute CHO ingestion resulting in increased CHO utilisation (Bosch et al., 1996), glycogen depletion resulting in increased contribution of protein to energy provision (Wagenmakers et al., 1991), and habitual high fat consumption resulting in increased contribution of fat to energy provision (Volek et al., 2016). Clearly, skeletal muscle is a major site of ketolysis under fasting conditions, but central to the rationale for exogenous ketone supplementation must be that

ketolysis increases during exercise, makes a meaningful contribution to energy provision, and can alter patterns of substrate utilisation.

The pioneering work of Hagenfeldt, Wahren and colleagues (Hagenfeldt & Wahren, 1968; Hagenfeldt & Wahren, 1971; Wahren et al., 1984) and Fery, Balasse and colleagues (Balasse et al., 1978; Fery & Balasse, 1983; Fery & Balasse, 1986; Fery & Balasse, 1988) established that KB disposal into human skeletal muscle is elevated as much as five-fold during exercise. This is generally reflected by a drop in KB concentrations soon after the onset of exercise, primarily βHB, concomitant with increases in KB oxidation in skeletal muscle and elevated metabolic clearance rate (MCR). MCR is a measure of the ability of tissues to remove ketones from the blood, analogous to arteriovenous difference per unit time, but measured during exercise is taken to represent an index of the ability of exercise to stimulate the capacity of working muscles to extract and utilise ketones (Fery & Balasse, 1983; Balasse & Fery, 1989). Because the stoichiometry of KB oxidation yields respiratory quotients of 1.00 and 0.89 for AcAc and βHB, respectively (Frayn, 1983), calculation of oxidation rates for KB from whole-body gas exchange data has not been routinely performed using methods that determine the relative contribution of CHO and fat oxidation. However, a recent attempt has been made (Cox et al., 2016) based on methods and assumptions described for KB utilisation during ketogenesis (Frayn, 1983). Previous to this, oxidation rates for KB have historically been derived from arteriovenous differences of radiolabelled KB across working muscles with rates calculated as a fraction of O_2 consumption or CO_2 production (Hagenfeldt & Wahren, 1968; Balasse et al., 1978).

Like CHO and fat utilisation, KB metabolism during exercise is influenced by a variety of factors including metabolic status (Wahren et al., 1984; Fery & Balasse, 1986), training status (Johnson & Walton, 1972; Rennie et al., 1974; Beattie & Winder, 1985), and the intensity of exercise (Cox et al., 2016). Given the aforementioned fibre type-specific

differences for activities of ketolytic enzymes, the muscle fibre type profile of the working muscle is also likely to be an important determinant of ketolysis during exercise. However, the most important determinant of KB metabolism during exercise is the degree of ketonemia, and the method by which this is achieved i.e. of endogenous or exogenous origin.

Ketone body metabolism during exercise under conditions of *endo*genous ketosis

Like KB metabolism in resting skeletal muscle, the relationship between concentration and oxidation or MCR is curvilinear (Balasse & Fery, 1989). At low ketonemia (<1.0 mM) such as that produced by an overnight fast, resting MCR is as much as four-fold greater than during prolonged fasting (Fery & Balasse, 1983). During prolonged exercise of low-to-moderate intensity after an overnight fast, MCR increases by 50% to 75% (Fery & Balasse, 1983; Fery & Balasse, 1986), which indicates that working muscle has an increased capacity to extract ketones from blood compared to rest. However, when ketonemia exceeds 2.5 mM such as that achieved by greater than 72 h of fasting, the exercise-induced rise in MCR is abolished (Fery & Balasse, 1986). Therefore, when ketosis is achieved by prolonged (>72 h) fasting there is a negligible contribution of KB oxidation to energy provision (Hagenfeldt & Wahren, 1971; Fery & Balasse, 1986), but after an overnight fast, the contribution ranges from 2 to 10% (Balasse et al., 1978; Fery & Balasse, 1983; Wahren et al., 1984). Under these conditions, the majority of energy provision in working muscle is from CHO and fat as classically described (van Loon et al., 2001). Moreover, unlike CHO and fat, there is progressive attenuation of the oxidation of KB with rising ketonemia, and thus the mobilisation of KB is not the factor limiting oxidation in skeletal muscle. This attenuation of exercise-stimulated MCR suggests either that above a threshold concentration the capacity for skeletal muscle to oxidise KB becomes saturated, and/or that hyperketonemia itself is a self-inhibitory factor (Balasse & Fery, 1989). Mechanistically, this is likely mediated either

through the inhibition of OXCT by elevated AcAc, and/or via FFA-mediated inhibition of ketolysis (Robinson & Williamson, 1980). This regulation is critical in the starvation response because the capacity of the liver to produce KB closely matches the requirements of the brain to utilise KB as an energy source (Robinson & Williamson, 1980). Therefore, excessive oxidation by working muscle would threaten survival, whereas it's inhibition spares circulating substrate for the brain (Hagenfeldt & Wahren, 1971; Fery & Balasse, 1983).

Ketone body metabolism during exercise under conditions of *exo*genous ketosis

The aforementioned self-inhibitory effect of rising ketonemia underscores a key methodological issue when considering KB metabolism in skeletal muscle, namely the method of achieving ketosis. While fasting of various durations is a widely used model of ketosis, acute nutritional ketosis relevant to sports performance would be achieved with replete glycogen stores, and in the absence of prolonged elevations in FFA and KB concentrations that would likely impair KB oxidation rates through these mechanisms. To our knowledge, only two studies have addressed this convincingly by examining effects of exercise on KB metabolism without interference from the various hormonal and metabolic perturbations associated with prolonged fasting or diabetes (Fery & Balasse, 1988; Cox et al., 2016).

In the former study (Fery & Balasse, 1988), infusion of sodium AcAc after an overnight fast achieved KB concentrations of ~6 mM (βHB ~3.5 mM, AcAc ~2.5 mM) at the onset of 2 h of exercise at ~52%$\dot{V}O2_{peak}$. Notably, AcAc did not change during exercise whereas βHB declined throughout exercise to be reduced by ~2 mM at the end of exercise. This coincided with a progressive rise in MCR throughout exercise peaking at ~75% higher than rest at the end of exercise. In contrast, this effect was abolished with similar ketonemia in three to five day fasted participants. Importantly, although the inhibition of KB oxidation

by hyperketonemia is present during exogenous ketosis, an "auto-amplification" was noted that is not present in fasting ketosis i.e. the initial rise in MCR induced by exercise causes a reduction in concentration which, in turn, provokes a further rise in MCR and so on. Additionally, the threshold concentration at which hyperketonemia inhibits MCR was higher in exogenous ketosis than in fasting ketosis. However, in terms of contribution to energy provision, this ultimately only resulted in a 2% contribution over the 2 h exercise bout. Nevertheless, plasma lactate concentrations did not rise during exercise after AcAc infusion compared to a ~1 mM rise in the fasted participants, which suggests that despite a modest contribution to energy provision, exogenous ketosis can impact on metabolic processes during exercise.

Metabolic effects of ingesting ketone salts and ketone esters at rest and during exercise

Ketone salts at rest

Ingestion of 1.6 and 3.2 $mM.kg^{-1}$ body mass in the form of a sodium/potassium racemic KS elevates whole blood βHB concentrations to ~0.8 mM and 1.0 ± 0.1 mM, respectively with concentrations peaking between 60-90 min (Stubbs et al., 2017). Similarly, ingestion of 0.5 $g.kg^{-1}$ body mass sodium/calcium KS, providing ~0.31 $g.kg^{-1}$ body mass βHB elevates plasma βHB concentrations to 0.60 ± 0.30 mM after 2.5 h, after which concentrations returned to baseline over 3 h (Fischer et al., 2018). Elevations in βHB concentrations via KS ingestion coincide with decreases in plasma FFA, triglycerides and glucose, and an elevation in plasma insulin, which may be accounted for by the small amount of carbohydrate used to sweeten the KS (Stubbs et al., 2017). KS ingestion does not alter blood pH or plasma lactate, but reduces plasma potassium concentrations and increases sodium and chloride concentrations (Fischer et al., 2018). L-βHB concentrations are elevated

to ~2.0 mM with the ingestion of KS (Stubbs et al., 2017), suggesting racemic KS products are more effective at raising the L-enantiomer than the D-enantiomer of βHB.

Ketone salts during exercise

Acute ingestion of one serving KS elevates whole blood βHB concentrations between 0.5-0.8 mM (O'Malley et al., 2017; Rodger et al., 2017; Waldman et al., 2018). Given the lower dosage employed and the discrepancy in βHB measurement between handheld monitors and laboratory methods, it is a salient question whether these measurements in whole blood are accurate. Serving sizes have ranged from 11.38 g βHB to 23.4 g D/L-βHB. This modest elevation in βHB concentrations is accompanied by an ~10% decrease in plasma glucose during sub maximal exercise (O'Malley et al., 2017), however, this decrease is not always observed (Rodger et al., 2017; Waldman et al., 2018). During submaximal exercise, plasma lactate is not affected by acute KS ingestion (Rodger et al., 2017; O'Malley et al., 2017; Waldman et al., 2018). Ingestion of KS lowers RER during exercise (O'Malley et al., 2017) given the respiratory quotient of AcAc is 1.00, similar to that of CHO it would be expected to rise when compared to a non-caloric placebo, so more work is needed as to whether training status or a larger dose may have a further effect on RER during exercise. Studies using the manufacturers recommended dose of KS do not report incidences of gastrointestinal distress. However, given the modest elevation in βHB concentrations during exercise with these dosages, increasing the dose is required to achieve βHB >1.0 mM, which may cause gastrointestinal distress, possibly due to acute hyperosmotic load.

Increases in heart rate (HR) have been observed with βHB infusions (Gormsen et al., 2017; Svart et al., 2018) but this is not observed with KS ingestion (Waldman et al., 2018). It is likely the sodium load in KS is causing this elevation in HR as it is not observed with either acute KME or KDE supplementation. Acute sodium ingestion can elevate blood

pressure (Farquhar et al., 2005), and a similar sodium load delivered as sodium bicarbonate results in an elevation in HR of ~10 bpm during moderate intensity activity (Kahle et al., 2013).

Ketone esters at rest

In the fasted state, ingestion of 573 mg.kg^{-1} body mass KME elevates plasma βHB concentrations to 3.5 ± 0.3 mM at 10 min, reaching ~6.0 and ~6.5 mM after 40 and 70 min, respectively (Cox et al., 2016). Ingestion of 482 mg.kg^{-1} KME elevates blood βHB concentrations to ~3.2 mM after 30 min, thereafter concentrations decrease during a 2 h oral glucose tolerance test (Myette-Côté et al., 2018). Whole blood βHB concentrations peaked at 2.8 ± 0.2 mM 60 min following the ingestion of 282 mg.kg^{-1} KME and at ~1.5 mM 20 min following ingestion of 141 mg.kg^{-1} (Stubbs et al., 2017). These results demonstrate a dose response in βHB concentrations following KME ingestion, with elevating concentrations following increasing dosages in the fasted state. Feeding status alters the βHB response to KME ingestion, as a prior breakfast (600 kcal; macronutrient ratio carbohydrate: protein: fat of 2:1:1) attenuates the elevation in whole blood βHB by ~1.0 mM (Fed: ~2.0 mM; Fasted ~3.0 mM), reducing the βHB area under the curve by 27% following 395 mg.kg^{-1} body mass KME (Stubbs et al., 2017). Conversely, ingestion of 500 mg.kg^{-1} body mass KDE elevates serum βHB to ~0.4 mM 60 min after ingestion of the initial bolus (Leckey et al., 2017), a major difference between the two ketone esters. The AcAc response to KME and KDE ingestion are similar, with concentrations elevated to ~0.3 mM 60 min after KDE ingestion (Leckey et al., 2017) and ~0.7 mM after KME ingestion (Stubbs et al., 2017). The elevation in βHB concentrations with KME coincides with decreases in plasma glucose, FFA and triglycerides (Cox et al., 2016; Stubbs et al., 2017; Myette-Côté et al., 2018).

A secondary mechanism for the glucose lowering effect of ketone esters has been proposed (Myette-Côté et al., 2018); ingestion of KME resulted in a 44% reduction in FFA AUC over the course of a 2 h oral glucose tolerance test, with the authors suggesting the anti-lipolytic action of KB may be responsible for this increase insulin sensitivity. The insulinotropic effects of KB in humans are evident (Balasse et al., 1970; Miles et al., 1981) but is frequently not observed (Balasse & Ooms, 1968; Balasse & Neef, 1975; Balasse, 1979; Fery & Balasse, 1980; Beylot et al., 1986; Nair et al., 1988; Mikkelsen et al., 2015); it is suggested that the insulinotropic action of ketone bodies occur under conditions where the ketone body concentration is raised above 2.0 mM, and when this rise occurs abruptly (Balasse & Fery, 1989). Given the elevation in circulating βHB observed with KME ingestion, it may have an insulinotropic effect. In the context of ketone ester ingestion, insulinotropic is defined as an increase in circulating insulin concentrations with exogenous ketone supplement plus nutrient condition being greater than nutrient alone. In the fasted state, ingestion of KME causes a small elevation in insulin (Stubbs et al., 2017; Stubbs et al., 2018), but is ~20% to 40% of the response to isocaloric carbohydrate ingestion. When KME is coingested with carbohydrate, there is no difference in circulating insulin concentrations compared to carbohydrate alone (Stubbs et al., 2017; Myette-Côté et al., 2018; Cox et al., 2016).

Ingestion of 1.9 $kcal.kg^{-1}$ body mass of KME (102-210 kcal; 21-43 g βHB) elevated whole blood βHB concentrations to 3.3 ± 0.2 mM after 1 h while attenuating the rise in circulating insulin and ghrelin compared to an isocaloric dextrose drink. This attenuation in plasma ghrelin resulted in a decrease in feelings of hunger, desire to eat and increased levels of fullness, suggesting KME may aid dietary strategies aimed at weight loss (Stubbs et al., 2018)

Ketone esters during exercise

Many of the responses seen at rest with exogenous ketone ingestion are prevalent during exercise. In one of a series of experiments, ingestion of 573 $mg.kg^{-1}$ body mass KME prior to 45 min cycling at 40% and 75% peak power output (W_{max}) elevated βHB concentrations to ~4.0 mM and ~3.0 mM respectively and βHB contributed 18% and 16% towards total oxygen consumption. The exercise induced rise in plasma lactate was attenuated by ~50% during 1 h cycling at 75% W_{max} compared to isocaloric carbohydrate ingestion (Cox et al., 2016).

The glucose lowering effect of ketone esters during exercise is well-demonstrated (Cox et al., 2016; Leckey et al., 2017; Vandoorne et al., 2017; Myette-Côté et al., 2018). This effect is caused by reduced hepatic gluconeogenesis (Mikkelsen et al., 2015) and occurs independently of differences in circulating insulin between conditions (Vandoorne et al., 2017; Myette-Côté et al., 2018).

Ketone ester ingestion prior to exercise attenuates the exercise induced increase in plasma lactate (Cox et al., 2016; Leckey et al., 2017). One of the main proposed benefits of exogenous ketones is a 'glycogen sparing' mechanism that will confer an advantage during periods of competition that are high intensity in nature and carbohydrate dependent (Pinckaers et al., 2016). Carbohydrate utilisation is reduced during submaximal exercise with KME ingestion in the fasted state (Cox et al., 2016), and it remains to be seen whether this sparing of carbohydrate can be overcome later in exercise or whether it will impair performance if the exercise duration is sufficiently extended. When compared to the effect of KS ingestion on plasma lactate, βHB concentrations between ~1.0 to 2.0 mM may be required to elicit an attenuation in plasma lactate concentrations.

The exercise-induced rise in FFA and glycerol is blunted with KME ingestion during 60 min cycling at 75%W_{max} compared to isocaloric fat and carbohydrate ingestion. Plasma

insulin is elevated to ~7 $mU.L^{-1}$ following KME ingestion but remains lower than isocaloric carbohydrate ingestion. KME increases the contribution of IMTG to total energy provision during exercise and results in a preservation of muscle glycogen (Cox et al., 2016).

Ketone body metabolism after exercise: post-exercise ketosis

Despite the aforementioned decline in KB concentrations at the onset of exercise, this pertains to situations where exercise has begun during hyperketonemia (Balasse et al., 1978; Fery & Balasse, 1983; Fery & Balasse, 1988; Cox et al., 2016). In the post-absorptive state, the pattern generally observed is for KB concentrations to rise gradually during prolonged exercise up to 0.2 to 0.4 mM, after which time post-exercise ketosis (PEK) of 0.3 to 2.0 mM is observed for several hours into recovery (Koeslag, 1982). Explained in terms of plasma kinetics, at cessation of exercise, the rate of appearance of KB increases coincident with a decrease in MCR relative to rates present during exercise. MCR remains above resting values for several hours after exercise, but ketogenesis exceeds ketolysis during this period.

On a mechanistic level, regulation likely resides at several sites including malonyl CoA-mediated regulation of fat transport into hepatocytes via CPT-1 in addition to availability of Ac-CoA for ketogenesis, and oxaloacetate for the TCA cycle as classically described for ketogenic regulation. Because oxaloacetate is a product of pyruvate formed during glycolysis, reductions in glycolytic flux with low glycogen content after intense exercise result in oxaloacetate moving to cytoplasm for preferential use in gluconeogenesis, which allows diversion of Ac-CoA towards ketogenesis during the post-exercise period rather than citrate synthesis for the TCA cycle. Additionally, the actions of insulin and glucagon exert a strong influence through activation and inhibition, respectively, of Ac-CoA carboxylase (ACC), which catalyses the synthesis of malonyl CoA from Ac-CoA. When liver glycogen becomes depleted and glucagon:insulin ratio is elevated, the synthesis of malonyl

CoA is reduced, thereby relieving the inhibition of fat transport into hepatocytes, and resulting in elevated concentrations of Ac-CoA. These regulatory mechanisms are acutely sensitive to nutrient manipulations before and after exercise and to aerobic exercise training, given their respective influences on substrate availability and utilisation during exercise.

Modulation of post-exercise ketosis by aerobic exercise training and nutrition intervention

An attenuation of, or abolished, post-exercise ketosis has been consistently observed in rodents and humans in response to aerobic exercise in trained versus untrained individuals (Johnson et al., 1969; Johnson & Walton, 1972; Rennie et al., 1974), or after a period of exercise training (Rennie & Johnson, 1974; Beattie & Winder, 1984, 1985; Adams & Koeslag, 1988; Adams & Koeslag, 1989; Ohmori et al., 1990). The aforementioned enhanced ketolytic capacity and downregulation of ketogenic capacity by training may play a role in these observations, but the majority of this work has been performed in comparisons with the absolute exercise intensity and duration being the same for comparisons (reviewed in (Koeslag, 1982)). This is problematic because the relative exercise intensity is the key determinant of the metabolic and hormonal response to acute exercise e.g. catecholamine responses, FFA mobilisation, glycogen utilisation among others. When trained and untrained participants have performed exercise at a similar relative intensity, PEK is blunted but not abolished in trained individuals (Rennie et al., 1974). Moreover, in rodents when exercise is completed to exhaustion, i.e. the trained rats exercise for longer than untrained, βHB concentrations are ~two-fold higher at the exercise cessation in the trained group (Askew et al., 1975). These divergent findings are likely due to the degree of liver glycogen depletion that occurs (Adams & Koeslag, 1988), inasmuch as higher concentrations of resting liver glycogen and attenuated rates of depletion are a consequence of training (Baldwin et al., 1975).

Therefore, PEK is strongly influenced by nutrition manipulation. High CHO feeding prior to exercise attenuates PEK regardless of training status (Rennie & Johnson, 1974; Askew et al., 1975; Koeslag et al., 1980), and CHO restriction increases PEK (Impey et al., 2016). Glucose ingestion at 2 h into recovery (Koeslag et al., 1982; Carlin et al., 1987) and alanine during recovery (Koeslag et al., 1980; Koeslag et al., 1985; Carlin et al., 1987) attenuate PEK

, but the glucose effect is not seen when glucose is ingested immediately after exercise. Alanine ingestion increases mitochondrial oxaloacetate concentrations in liver, thereby allowing condensation with Ac-CoA and diversion away from ketogenesis. This suggests that the early PEK response is determined by the extent of liver glycogen depletion and reduced glycolytic flux, whereas several hours into recovery is under regulation by insulin and FFA concentrations related to nutrition intake.

Metabolic consequences of post-exercise ketosis during recovery: a role for exogenous ketones as a recovery aid?

The physiological role for PEK is likely to favour the replenishment of muscle glycogen, consistent with classically described metabolic actions of ketosis in the sparing of protein and CHO stores during times of low CHO availability. During the post-exercise recovery period, in contrast to the reliance on CHO metabolism during exercise, muscle glycogen resynthesis has a high metabolic priority and is facilitated by an increase in fat oxidation and sparing of CHO sources for energy provision (Kiens & Richter, 1998). A priority for muscle glycogen resynthesis over liver glycogen resynthesis is suggested to occur because in ancestral terms, a depleted liver is less of hindrance to intense exertion than depleted muscle (Adams & Koeslag, 1988). To this end, the priority for muscle glycogen resynthesis is observed even during CHO restriction (Adams & Koeslag, 1989), and is

achieved through non-CHO sources such as lactate and alanine being used for hepatic gluconeogenesis and redistribution to skeletal muscle (Fournier et al., 2002). The contribution of PEK may be via the ability of KB to inhibit glycolysis and increase the conversion of glucose to glycogen as demonstrated in rat skeletal muscle in vitro (Maizels et al., 1977), and a perfused heart model in dogs (Laughlin et al., 1994). This effect is likely mediated by inhibition of PDH and phosphofructokinase (PFK) by elevations in Ac-CoA and citrate formation, respectively, as a consequence of metabolism of AcAc in mitochondria (Randle et al., 1964; Maizels et al., 1977; Laughlin et al., 1994; Kashiwaya et al., 1997).

This raises the possibility that an optimal post-exercise recovery milieu exists that includes both CHO and ketones to enhance recovery of muscle glycogen. This is not possible by conventional nutrition strategies because elevations in glucose, lactate and alanine ultimately limit ketogenesis and PEK. The suggestion is that the co-ingestion of KME and CHO in a recovery protocol can confer a metabolic advantage.

Following exercise, glycogen re-synthesis and stimulation of muscle protein synthesis (MPS) are of high importance to improve recovery. Athletes are recommended to follow optimal carbohydrate and protein-based fuelling related to the type, timing and amount of carbohydrate and protein to facilitate this recovery process and prepare the next bout of exercise. Ingestion of carbohydrate at a rate of 1.2 $g.kg^{-1}$ body mass. h^{-1} during the first 4 h of recovery maximises the rate of muscle glycogen resynthesis; addition of 0.4 $g.kg^{-1}$ body mass. h^{-1} of protein added to suboptimal carbohydrate doses during recovery increases the rate of glycogen resynthesis compared to suboptimal carbohydrate alone (Beelen et al., 2010). When the time between exercise bouts is limited (< 8 h), nutritional strategies to augment glycogen resynthesis for subsequent exercise performance are a priority (Burke et al., 2017). During recovery from endurance exercise, the general pattern is a gradual rise in ketosis for several hours in the range of 0.3 to 2.0 mM, termed post exercise ketosis (Koeslag, 1982).

This rise in PEK is attenuated in aerobically-trained individuals when exercise is performed at the same absolute intensity, but not when performed at the same relative intensity (Johnson et al., 1969; Rennie and Johnson, 1974). Carbohydrate availability abolishes the rise in PEK during 3 h recovery following a mixture of high intensity and steady-state exercise, demonstrating the influence of circulating nutrients on ketosis (Impey et al., 2016). Similarly, alanine ingestion during recovery attenuates PEK (Koeslag et al., 1980; Carlin et al., 1987).

The physiological role for PEK is likely to favour the replenishment of muscle glycogen and enhancing MPS, consistent with the metabolic action of KB as a survival mechanism during periods of low carbohydrate availability. Resting muscle glycogen concentrations are not lower in 'fat adapted' ultra-endurance athletes following a high fat (70%) diet for 20 months; during and after 3 h submaximal running, the pattern of glycogen utilisation and recovery respectively was not different to a matched group of athletes following a high carbohydrate (55%) diet despite a larger contribution of fat towards total energy consumption (Volek et al., 2016). This is a pattern repeated in Alaskan sled dogs, who despite following a high fat/low carbohydrate diet show no cumulative signs of muscle glycogen depletion over several days of exercise or inability to oxidise carbohydrate (McKenzie et al., 2005; McKenzie et al., 2008). Glycogen replenishment following endurance exercise is facilitated by non-carbohydrate gluconeogenic sources, of which KB may be one. In a postabsorptive state, over 90% of gluconeogenesis is accounted for by gluconeogenic amino acids (alanine, glutamine), lactate and glycerol (Gerich et al., 2001), suggesting the preservation of muscle glycogen stores observed in fat-adapted humans and dogs is due to the increase in gluconeogenic substrate availability to the liver, preserving circulating glucose concentrations. KB act to inhibit glycolysis in rat skeletal muscle in vitro (Maizels et al., 1977), and a perfused heart model in dogs (Laughlin et al., 1994) and infusion of βHB stimulates muscle protein synthesis by 10% (Nair et al., 1988). These data would

suggest exogenous ketones could play in enhancing muscle glycogen re-synthesis and MPS in the recovery period after exercise.

In the fasted state, ingestion of 573 $mg.kg^{-1}$ body mass KME immediately after glycogen-depleting exercise alongside a 2 h hyperglycaemic clamp reportedly increases muscle glucose uptake by 32%, insulin concentrations two-fold and muscle glycogen concentrations by 50% compared to an isocaloric control (glucose only) drink (Holdsworth et al., 2017). Participants completed a glycogen-depleting bout of interval cycling lasting 115 ± 2 min. A 10 mM hyperglycaemic clamp was chosen to replicate circulating glucose concentrations during a practically-relevant nutrition intervention and KME or control drinks were provided immediately after exercise. Blood βHB concentrations peaked at 5.3 ± 0.5 mM during recovery and were 3.3 ± 0.2 mM at the end of the clamp. The stated 50% increase in muscle glycogen in the KME condition represents the absolute increase muscle glycogen after the clamp regardless of immediate post-exercise muscle glycogen concentrations (KET: 114 ± 23 $mM.kg^{-1}$ dry weight (DW); CON: 70 ± 23 $mM.kg^{-1}$ DW) i.e. muscle glycogen was decreased by 30% more in the KET condition than in the CON condition. At the end of the 2 h recovery phase, muscle glycogen concentrations during KME (132 ± 20 $mM.kg^{-1}$ DW) increased by 86% and during CON (94 ± 15 $mM.kg^{-1}$ DW) by 74%, representing a 12% increase in muscle glycogen re-synthesis with acute KME ingestion, which may be accounted for by the increase in caloric intake with KME (5 $kcal.\ g^{-1}$). The proposed mechanism for the enhanced muscle glycogen re-synthesis is stated to be the doubling of insulin concentrations with KME (KME: 31±5 $mU.L^{-1}$; CON: 16±3 $mU.L^{-1}$). However, early post-exercise muscle glycogen resynthesis appears to independent of circulating insulin, lasting 30-60 min when muscle glycogen concentrations are depleted below 150-200 $mM.kg^{-1}$ DW (Beelen et al., 2010), as is the case in this study.

Conversely, a separate study demonstrated that there is no effect on muscle glycogen repletion when 0.50 $g.kg^{-1}$ body mass KME is ingested immediately after exercise, followed by 0.25 $g.kg^{-1}$ body mass alongside 1.00 $g.kg^{-1}$ body $mass.h^{-1}$ carbohydrate and 0.30 $g.kg^{-1}$ body $mass.h^{-1}$ whey protein in the form of a recovery drink during a 5 h hour recovery (Vandoorne et al., 2017). This feeding regimen was chosen to elicit maximal rates of MPS (Morton et al., 2015) and muscle glycogen re-synthesis during the post-exercise recovery period (Burke et al., 2017). Participants were provided with a high carbohydrate (70%) breakfast and completed a glycogen-depleting bout of unilateral knee extensions. Whole blood βHB concentrations peaked at 4.3 ± 0.5 mM at 4 h of recovery. Unilateral exercise depleted muscle glycogen concentrations by ~60% during both PLA and KME, reaching 170 ± 22 $mM.kg^{-1}$ DW and 183 ± 21 $mM.kg^{-1}$ DW, respectively. No difference in plasma insulin concentrations was observed during the 5 h recovery period, reaching ~25 $mU.L^{-1}$ at 90 and 300 min of recovery in KME and PLA conditions and blood glucose was ~1.0 mM lower during KME during the final 4 h of recovery. Differences in study design may explain the contrasting findings of these two studies, e.g. different exercise modalities in cycling vs. unilateral exercise, the different method of elevating plasma glucose availability (hyperglycaemic clamp vs. powdered drinks), the different lengths of recovery period, and the magnitude of glycogen depletion.

Regarding anabolic signalling in skeletal muscle, ingestion of KME alongside optimal post-exercise carbohydrate and protein fuelling increased the phosphorylation of proteins involved in the mammalian target of rapamycin (mTORC1)-regulated muscle protein synthesis cascade. In vivo phosphorylation of S6K1 increased ~two-fold and 4E-BP1 increased 60% with in the KME condition compared to placebo ingestion. In vitro, the addition of βHB + AcAc (4.0 + 1.4 mM, respectively) to 1.5 mM leucine increased S6k1 phosphorylation ~three-fold and 4E-BP1 by 51% in skeletal muscle cells. Moreover, the

addition of βHB + AcAc to 1.5 mM leucine increased the rate of MPS by two-fold compared to leucine alone. However, KB were not added to the higher leucine concentration of 5.0 mM, which alone stimulated MPS to a higher extent than a combination of βHB, AcAc and 1.5 mM leucine (Vandoorne et al., 2017). Therefore, it remains to be explored whether AcAc and βHB have anabolic effects similar to leucine alone, despite earlier reports of a 10% in MPS after βHB infusion in humans (Nair et al., 1988).

To examine the anticatabolic effect of KB, infusion of 2.4 $mL.kg^{-1}$ body mass. h^{-1} D/L-βHB increased plasma βHB concentrations to 3.5 mM in a LPS stimulated inflammatory model in humans (Thomsen et al., 2018). Compared to FFA, infusion of D/L-βHB reduced forearm phenylalanine release by ~70%, reflecting a net decrease in muscle protein loss and whole body phenylalanine-to-tyrosine degradation was reduced. The insulin-Akt-mTOR and p70 S6K phosphorylation was unaffected by D/L-βHB infusion. These results suggest an anti-catabolic effect of βHB at a muscle and whole body level, rather than a direct anabolic effect. Direct comparisons between βHB and amino acids and simultaneous administration of both substrates is required before any statement can be made about the efficacy of βHB as a treatment in muscle wasting/catabolic models.

These preliminary data suggest that when undertaking best practice recovery guidelines, the addition KME confers no benefit to glycogen re-synthesis, but may enhance MPS. However, it remains to be seen whether addition of KS, KDE or KME to suboptimal delivery of carbohydrate post exercise may, like protein, aid with glycogen re-synthesis.

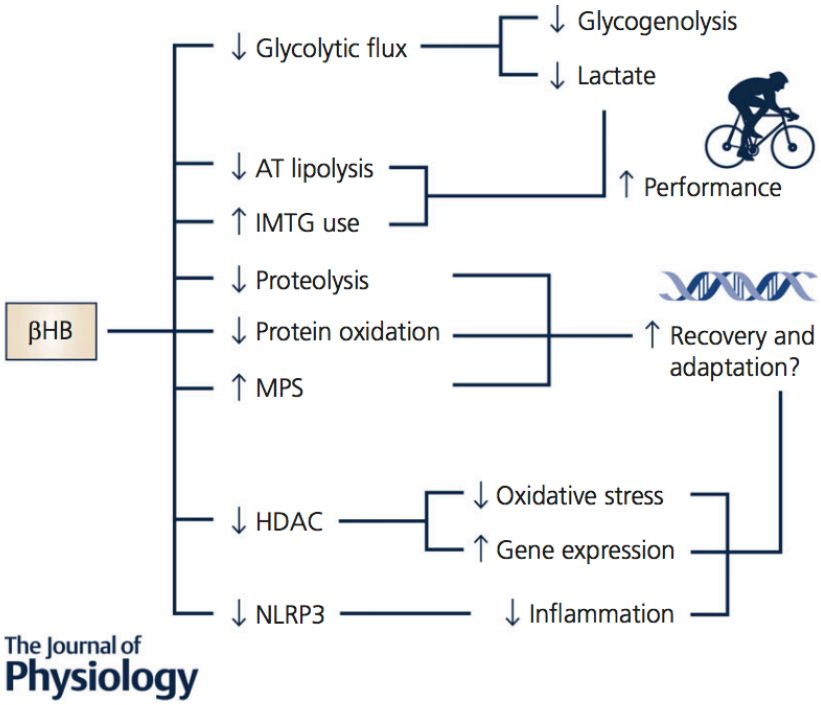

Figure 2.6 βHB as a metabolic regulator and signalling metabolite
Effects of elevating βHB through acute nutritional ketosis may be mediated by acute regulation of substrate utilisation that may enhance performance, and /or possibly through regulation of recovery and adaptive processes related to inflammation, oxidative stressand changes in gene expression.

Effects beyond fuelling: βHB as a HDAC inhibitor

As investigative techniques in molecular biology evolve, so too does our appreciation of how complex integrative signalling networks regulate skeletal muscle adaptation in response to stimuli such as nutrient manipulation and exercise training (Egan & Zierath, 2013). Previously considered relatively inert outside their primary metabolic function, numerous substrates and metabolites are emerging as important regulators of intracellular signalling and tissue adaptation (Hashimoto et al., 2007; Gao et al., 2009; Morton et al., 2009; Roberts et al., 2014). Noteworthy for the present review is the recent identification of AcAc as a regulator of skeletal muscle satellite cell proliferation and muscle regeneration (Zou et al., 2016), and βHB as an inhibitor of HDACs (Shimazu et al., 2013) and the NLRP3 inflammasome (Youm et al., 2015). The latter observations are a consequence of βHB, in

essence, acting as a signalling metabolite to regulate gene expression and metabolic processes (Figure 2.6).

Histone acetyltransferases (HATs) and HDACs are enzymes that facilitate the addition or removal, respectively, of acetyl moieties from specific lysine residues on histones and target proteins (McKinsey et al., 2001). In general, hyperacetylation of histone tails induces transcriptional activation while hypoacetylation is associated with transcriptional repression. Class IIa HDACs (HDAC4, -5, -7 and -9) are highly expressed in skeletal muscle (McKinsey et al., 2001) and their function is responsive to both aerobic endurance exercise in humans (McGee et al., 2009; Egan et al., 2010) and nutritional intervention in rodents (Gao et al., 2009; Shimazu et al., 2013). An acute bout of aerobic exercise increases class IIa HDAC phosphorylation and subsequent nuclear exclusion, thus inhibiting HDAC-mediated repression of specific exercise-responsive genes such as GLUT4 and PGC-1α (McGee & Hargreaves, 2004; McGee et al., 2009; Egan et al., 2010). This suggests that compounds that inhibit or disrupt HDAC inhibition could be used to mimic or enhance adaptations to exercise.

Regulation of HDAC activity by nutrients including butyrate and βHB has also been established (Gao et al., 2009; Shimazu et al., 2013). Butyrate, a short chain fatty acid formed via the fermentation of indigestible dietary fibres by microbial species in the gut, is a potent inhibitor of HDAC activity (Gao et al., 2009). Mice supplemented with sodium butyrate are resistant to diet-induced obesity, and have elevations in markers of skeletal muscle mitochondrial biogenesis analogous to exercise effects (Gao et al., 2009). βHB is structurally similar to butyrate, and although not as potent as butyrate, also inhibits HDAC class I and II activity in a dose-dependent manner and supressed oxidative stress responses (Shimazu et al., 2013). Importantly, HDAC inhibition by βHB both in vitro and in vivo is evident at physiologically-relevant concentrations of βHB i.e. 1 to 4 mM, which is similar to those attained during fasting, PEK and exogenous ketone ingestion (Figure 2.6) (Clarke et al., 2012;

Kesl et al., 2016). However, although the inhibitory effects were observed in multiple tissues, they remain to be confirmed in skeletal muscle. If confirmed, it will be intriguing to explore whether, apart from the aforementioned ergogenic effects, exogenous ketone supplementation complements exercise-mediated adaptive changes associated with modulating HDAC function.

Effects on physical performance

Ketone esters

The aforementioned work by Cox and colleagues at the University of Oxford remains the seminal study demonstrating the potential benefits of exogenous ketones on endurance performance (Cox et al., 2016). Ingestion of 573 mk.kg^{-1} body mass KME improved 30 min max distance cycling time trial performance by ~2% (411 ± 162 m), following a 1 h pre-load at 75% $W_{max.}$ This improvement coincided with a ~1.5-2.0 mM reduction in plasma lactate and a reduction in plasma glucose. The drinks consumed in these trials were isocaloric, meaning performance improved despite receiving less carbohydrate throughout the KME arm of the trial (Cox et al., 2016). Since then, several reports on the physical performance effects of acute exogenous ketones ingestion have emerged (O'Malley et al., 2017; Rodger et al., 2017; Leckey et al., 2017; Waldman et al., 2018). These studies all use exogenous ketone supplementation, but in different forms, and it is important to distinguish between the different forms given that each have different metabolic effects that can potentially alter performance.

Ingestion of 500 mg.kg^{-1} body mass KDE impaired performance during an ecologically-valid 31.2 km time trial in professional cyclists (Leckey et al., 2017). Participants ingested the bolus as two 250 mg.kg^{-1} body mass aliquots 50 and 30 min prior to the time trial, having consumed a high carbohydrate breakfast (2 g.kg^{-1} body mass) on the

morning of each trial. Participants received 200 mg caffeine, a 6% carbohydrate sports drink and a caffeine gel during the trial to mimic the real-world practice in professional cycling. Performance was impaired by 2 ± 1%, (58.2 s; ES -0.42 small), explained by a 3.7% reduction in power output and was accompanied by a high prevalence of gastrointestinal distress among participants in the KDE condition. The lower plasma βHB concentrations in the KDE study makes it difficult to compare the performance findings with the KME work (Cox et al., 2016). Plasma βHB reached ~0.4 mM pre-time trial, suggesting KDE at this dose is currently ineffective at achieving nutritional ketosis (>0.5 mM βHB), a problem complicated by the reports of high prevalence of gastrointestinal distress.

To date, ketone ester work has focused on endurance athletes due to the proposed glycogen sparing mechanism of elevated circulating βHB concentrations (Cox et al., 2016). Sport nutrition guidelines for team sports promote high dietary carbohydrate intake prior to and during competition to maximise muscle glycogen stores with a view to enhancing performance (Burke et al., 2006). Therefore, nutritional strategies that could attenuate the reduction of muscle glycogen during match play (Mohr et al., 2003) is of value to scientists and practitioners. Whether ketone ester ingestion benefits team sport activity, and indeed most other sporting contexts, remains to be confirmed.

Ketone salts

There are three reports on exercise performance effects of acute KS ingestion (O'Malley et al., 2017; Rodger et al., 2017; Waldman et al., 2018). These studies use a variety of doses of KS. Ingestion of two servings of a commercially available KS (KetoForce), providing 11.7 g D/L-βHB per serving elevated whole blood βHB concentrations to 0.6 ± 0.2 mM but did not improve mean power output during 4 min maximal performance test in highly trained cyclists (KS: 364 ± 56 W; PLA: 355 ± 46 W) (Rodger et al., 2017). Similarly,

ingestion of one serving of KS (PerfectKeto) elevated whole blood βHB concentrations to 0.53 ± 0.19 mM but had no effect on mean power output (KS: 715 ± 94 W; PLA: 714 ± 93 W) or peak power output (KS: 969 ± 157 W; PLA: 955 ± 151 W) during 4x15 s maximal sprints on a cycle ergometer (Waldman et al., 2018). Ingestion of 0.3 $g.kg^{-1}$ body mass KS (KetoForce) 50 min prior to exercise elevated blood βHB concentrations to ~0.8 mM and lowered mean power output during a 150 kJ time trial (~10 km) (-7%, -16W) and resulted in a longer time to finish (KS: 711 ± 137 s; PLA: 665 ± 120 s) (O'Malley et al., 2017).

Overall, KS only modestly elevate βHB concentrations into the range of 0.3-0.8 mM during exercise, all measured by handheld monitors. Given the described overestimation of βHB concentrations by handheld monitors compared to gold-standard laboratory-based assays, it is likely βHB was lower than described in these works on KS ingestion and exercise performance. Secondly, elevation of βHB concentrations is postulated to impair high intensity exercise performance through an inhibition of glycolytic flux via inhibition of PDH and phosphofructokinase (PFK) by increases in NADH:NAD+, acetyl-CoA:CoA or citrate. The performance measures used in these studies were high intensity in nature , which are unlikely to benefit from exogenous ketone supplementation.

Effects on cognitive performance

The primary physiological role of KB is to provide glucose-dependent organs with a usable source of carbon during periods of low carbohydrate availability. FFA acids are unable to cross the blood-brain barrier and must be converted to KB in order to provide energy to the brain under these circumstances (Owen et al., 1967). KB account for ~60% of the brain's energy demands after 5-6 weeks of starvation in obese patients (Owen et al., 1967). After short term (3.5 d), brain glucose metabolism is reduced to ~70% of control values, measured

by positron emission tomography, and coincides with an increase in KB oxidation in compensation as an alternative fuel source to glucose (Hasselbalch et al., 1994).

Elevations in circulating KB concentrations have marked effects on brain energy metabolism. Elevating βHB concentrations to 4.0-8.0 mM by means of chronic fasting causes a 50% reduction in brain glucose MCR (Zhang et al., 2013). Moreover, infusion of D/L-βHB increased plasma D-βHB concentrations to 5.5±0.4 mM (similar to that achieved by prolonged fasting) and this coincided with a 14% reduction in cerebral glucose utilisation, a 30% increase in cerebral blood flow and unchanged oxygen consumption (Svart et al., 2018). The authors suggest may reduce oxidative stress, explaining the well-established neuroprotective properties of KB (Svart et al., 2018). This work was performed in the elderly, with similar results observed in young subjects (Hasselbalch et al., 1996) and is not related to the age-related decline in glucose utilisation.

Exogenous ketones may also play a role in improving cognition in Alzheimer's Disease (AD) and during mild cognitive impairment (MCI) by helping to overcome the characteristic brain energy deficit (Veech, 2004). During AD and MCI, brain glucose uptake is impaired while uptake of KB is unchanged and the elevation of KB through oral medium chain triglyceride supplementation improves cognitive outcomes in these populations (Reger et al., 2004; Krikorian et al., 2012; Cunnane et al., 2016).

Similarly, glucose uptake and oxidation in the brain is reduced in an exercise intensity-dependent manner, as evidenced by a reduction of ~30% when exercising for 35 min at ~75% compared to 30% $\dot{V}O2_{peak}$ (Kemppainen et al., 2005). Conversely, the uptake and oxidation of circulating lactate by the brain increases with increasing exercise intensities paralleling the downregulation of carbohydrate oxidation. Under resting conditions the uptake and oxidation of lactate is minimal but >4.0 mM the brain goes from a state of lactate release to uptake, related to an increase in arterial concentrations (Rasmussen et al., 2011). At

higher lactate concentrations, lactate may replace glucose as a source for the brain by up to 25% (Quistroff et al., 2008; Rasmussen et al., 2011). The point to be made here is that the brain can oxidise circulating substrates in an arterial concentration-dependent manner. AD, MCI and intense exercise are all characterised by a decrease in brain glucose oxidation that is compensated for by other substrates capable of crossing the blood brain barrier. Elevations in βHB concentrations via ketogenic supplementation can compensate for the deficit in glucose oxidation in AD and MCI (Cunnane et al., 2016), so it is tempting to speculate whether exogenous ketones may play a similar role in exercise contexts.

These works suggest that elevating circulating KB concentrations reduces the brain's reliance on glucose for fuel provision. Exercise places a high demand on whole body carbohydrate stores, and nutritional interventions that ensure normal brain energy homeostasis warrant investigation. There is no clear relationship between anaerobic maximal bouts of exercise and cognitive processes, while aerobic exercise lasting <90 min exerts a selective influence on cognition, including improvements in complex problem solving and attentional processes that are involved in response inhibition (Tomporowski, 2003). There is a facilitating effect of physical activity and cognitive performance, with the majority of work on arousal and reaction time, possibly due to elevated levels of cortical catecholamines. Much of this work has been performed using low intensity exercise at 40% to 60% $\dot{V}O2_{peak}$ (Davranche et al., 2006), while both acute and chronic physical activity improves executive function (Best, 2012). It is important to be cognisant of the selective effects of exercise on different aspects on cognitive function being measured in nutritional supplement trials. Submaximal exercise of >2 h in duration that depletes energy (carbohydrate) stores and dehydrates the athlete is more likely to negatively impact cognitive function (Cian et al., 2000; Cian et al., 2001). If there is a central effect of KB during exercise, it may be that KB

could help to conserve cognitive function during endurance exercise by alleviating the reliance on glucose as a fuel source, and attenuating the decline in brain energy stores.

Work on ketone ester ingestion, cognitive performance and brain metabolism is focused in rodents using KME (Murray et al., 2016; Pawlosky et al., 2017) and KDE (Ciarlone et al., 2016; Ari et al., 2016; Kovács et al., 2017). Chronic feeding of 2.6 $g.day^{-1}$ KME for 5 days improved decision-making and reduced time to completion of a radial maze task by 38% versus rats fed an isocaloric high carbohydrate diet (Murray et al., 2016). Concentrations of cortical and hippocampal glycolytic intermediates is lower following chronic treatment of KME and cortical citrate synthase concentrations are significantly higher, while citrate synthase, isocitrate, alpha-ketoglutarate, succinate, fumarate and acetyl Co-A were all elevated after chronic ingestion of KME (Pawlosky et al., 2017). These changes coincided with a significantly more energetic ΔG in the hippocampus of KME-fed rats. Chronic KDE ingestion decreases audiogenic and chemically-induced seizure activity in mice when βHB concentrations are elevated to between 0.8-1.0 mM during 8 wk of treatment. (Ciarlone et al., 2016). Furthermore, chronic KDE administration by intra-gastric gavage decreases absence epileptic activity when βHB concentrations are elevated to ~1.8 mM at the end of 1 wk of treatment (Kovács et al., 2017). These results are explained by an increase in GAD65/67 hippocampal protein expression, which code for GABA, with this elevation being antithetic to the reduction in GABA that contributes to seizure activity.

The cognitive effects of exogenous ketones are underexplored in humans with only one study incorporating cognitive tasks (Waldman et al., 2018). Following acute ingestion of KS, participants completed a 5-min reaction test during their warm up and immediately following 4x15 s anaerobic Wingate sprints on a cycle ergometer. No effect on reaction time was observed after the anaerobic activity despite a higher fatigue index in the KS condition (KET: 32.3 ± 13.9 W/s; PLA: 29.2 ± 12.6 W/s). However, a learning/order effect is noted by

the authors for hits and misses on the same test with repeated testing (Waldman et al., 2018). This protocol may also not have been long enough to cause a reduction in whole body energy stores or dehydration to cause substantial decline in cognitive function (Tomporowski, 2003), and would seemingly agree that short duration exercise has little impact on cognitive function. Like physical performance, this work suggests low concentrations of βHB (<1.0 mM) may not effect cognitive performance in this high intensity exercise context. Whether ketone ester ingestion has an effect on cognitive performance in endurance and high intensity exercise challenges remains to be studied.

State-of-the-art in exogenous ketone supplementation and exercise performance

Exogenous ketone supplements come in multiple forms; racemic ketone salts, a (R)-3-hydroxybutyl (R)-3-hydroxybutyrate ketone monoester, and a R,S-1,3-butanediol acetoacetate diester. Each of these supplements elevates βHB concentrations but differ in their effect on the metabolic response to exercise, and possibly exercise performance. There is a strong rationale for the performance-enhancing benefits of exogenous ketone in sport and the same is true for performance impairment, much of which would depend on the supplement being consumed and the nature of the exercise challenge. The (R)-3-hydroxybutyl (R)-3-hydroxybutyrate ketone monoester is the only supplement shown to be beneficial to endurance performance in cyclists, hypothesised to result from a 'glycogen sparing' effect. Whether this ergogenic effect will be observed in other sporting contexts, namely high intensity intermittent team sports, that rely on a high rate of carbohydrate oxidation for sustaining performance, and other modalities of endurance exercise (i.e. running) remains to be determined. Given the role of KB in fuel provision to the brain during short and long term starvation, the effect of exogenous ketone supplements on aspects of cognitive performance, including reaction time, sustained attention, and decision making is of interest.

Chapter 3

Evans M, Patchett E, Nally R, Kearns R, Larney M, Egan B (2018) Effect of acute ingestion of β-hydroxybutyrate salts on the response to graded exercise in trained cyclists. *European Journal of Sport Science* 18(3):376–86.

Abstract

Introduction: Acute ingestion of ketone salts induces nutritional ketosis by elevating β-hydroxybutyrate (βHB), but few studies have examined the metabolic effects of ingestion prior to exercise. **Methods:** Nineteen trained cyclists (12 male, 7 female) undertook graded exercise (8 min each at ~30%, 40%, 50%, 60%, 70%, and 80% $\dot{V}O2_{peak}$) on a cycle ergometer on two occasions separated by either 7 or 14 days. Trials included ingestion of boluses of either (i) plain water (3.8 mL kg body mass^{-1}) (CON) or (ii) βHB salts (0.38 g kg body mass^{-1}) in plain water (3.8 mL kg body mass^{-1}) (KET), at both 60 min and 15 min prior to exercise. **Results:** During KET, plasma βHB concentrations increased to 0.33 ± 0.16 mM prior to exercise and 0.44 ± 0.15 mM at the end of exercise (both $p < .05$). Plasma glucose was 0.44 ± 0.27 mM lower ($p < .01$) 30 min after ingestion of KET and remained ~0.2mM lower throughout exercise compared to CON ($p<.001$). Respiratory exchange ratio (RER) was higher during KET compared to CON ($p < .001$) and 0.03–0.04 higher from 30%$\dot{V}O2_{peak}$ to 60%$\dot{V}O2_{peak}$ (all $p < .05$). No differences in plasma lactate, rating of perceived exertion, or gross or delta efficiency were observed between trials. Gastrointestinal symptoms were reported in 13 out of 19 participants during KET. **Discussion:** Acute ingestion of βHB salts induces nutritional ketosis and alters the metabolic response to exercise in trained cyclists. Elevated RER during KET may be indicative of increased ketone body oxidation during exercise, but at the plasma βHB concentrations achieved, ingestion of βHB salts does not affect lactate appearance, perceived exertion, or muscular efficiency.

Introduction

The relationship between energy provision and factors intrinsic and extrinsic to exercise is complex, but has traditionally focused on the relative contribution of carbohydrate and fat being regulated by the intensity and duration of an exercise challenge (Egan & Zierath, 2013). Many sports nutrition strategies are based around optimizing carbohydrate provision before and during performance (Burke, 2015), but there is increasing interest in alternative fuelling strategies, particularly in endurance sports, including low carbohydrate and ketogenic diets, and the use of exogenous ketone supplements (Burke, 2015; Evans, Cogan, & Egan, 2017).

Ketone bodies [namely β-hydroxybutyrate (βHB) and acetoacetate (AcAc)] are produced in the liver during periods of low glucose availability such as during fasting, starvation and ketogenic diets (Robinson & Williamson, 1980; Balasse & Fery, 1989; Laffel, 1999). Although principally acting as an alternative fuel source for the brain when glucose concentrations are diminished, ketone bodies are also used by skeletal muscle to provide up to 10% of energy during exercise in the fasted state (Franken, Neef & Balasse, 1974; Balasse, Fery, & Neef, 1978; Fery & Balasse, 1983; Fery, Wahren, Sato, Ostman, Hagenfeldt, & Felig, 1984). However, the direct contribution to energy provision may be secondary to the potential metabolic action of supplemental ketones. For instance, ketone bodies have wide-ranging metabolic effects on peripheral tissues such as glucose sparing, anti-lipolytic effects and stimulation of muscle protein synthesis (Maizels et al., 1977; Nair et al., 1988; Mikkelsen et al., 2015). During moderate intensity exercise, infusion of sodium AcAc after an overnight fast attenuates the rise in plasma lactate (Fery & Balasse, 1988), whereas sodium βHB infusion similarly alters the metabolic response to very intense exercise in rats (Kamysheva & Ostrovskaia, 1980), and ischemic forearm exercise in humans (Lestan et al., 1994).

Despite these observations, the potential performance benefits of ketone bodies have been unexplored until the recent emergence of exogenous ketone supplements in the form of ketone esters and ketone salts. For instance, acute ingestion of the (R)-3-hydroxybutyl (R)-3-hydroxybutyrate ketone monoester produced plasma βHB concentrations of ~3 mM during exercise, and improved 30 min time-trial performance by 2% in elite cyclists (Cox et al., 2016). Ketone esters are not commercially-available to date, but ketone salts represent a cheaper, more readily-available exogenous ketone supplement. These salts comprise of the free acid form of βHB buffered with sodium, potassium, and/or calcium salts, but are less effective at elevating plasma βHB concentrations compared to the ketone monoester (Stubbs et al., 2017). The effects of acute ketone salt ingestion on short-duration, high intensity exercise performance in human has been the subject of two recent reports (O'Malley et al., 2017; Rodger et al., 2017), both of which did not observe the performance benefits associated with the ketone monoester. Given that this is an emerging field of research, and to better understand the impact of ketone salt ingestion on responses across a range of exercise intensities, the purpose of the present study is investigate the effect of acute ingestion of a commercially-available βHB salt formulation on the metabolic and physiological responses to a graded submaximal exercise session in young, trained male and female cyclists. We hypothesise that ingestion of the βHB salt formulation will alter the metabolic response to exercise based on our pilot data collection.

Methods

Participants

Nineteen trained cyclists [12 male, 7 female (Table 3.1)] gave written informed consent to participate after written and verbal explanation of the procedures. Ethical approval (permit number: LS-15-82-Evans-Egan) was obtained from the University College Dublin Research Ethics Committee. All participants were active in regular cycling training (≥6

sessions per week) and competition in road, time-trial, and/or triathlon disciplines, and had been competing in their respective discipline for at least one calendar year.

Table 3.1 Participant anthropometrics and fitness profile

	Whole cohort (n = 19)	**Males** (n = 12)	**Females** (n = 7)	**Males vs. Females** P value
Age (y)	26.8 ± 7.6	25.1 ± 6.2	30.3 ± 8.8	0.148
Height (m)	174.3 ± 8.9	178.7 ± 7.4	166.7 ± 5.7	0.002
Body mass (kg)	69.0 ± 9.7	73.8 ± 6.8	60.9 ± 8.5	<0.001
Body fat (%)	17.6 ± 6.8	13.7 ± 3.9	24.3 ± 5.0	<0.001
FFM (kg)	57.7 ± 10.5	64.3 ± 5.9	46.4 ± 5.3	<0.001
W_{max} (W)	325 ± 67	368 ± 40	251 ± 23	<0.001
LT (W)	245 ± 59	278 ± 41	187 ± 36	<0.001
$\dot{V}O2_{peak}$ ($L \cdot min^{-1}$)	4.3 ± 8.5	4.8 ± 4.3	3.3 ± 4.3	<0.001
$\dot{V}O2_{peak}$ ($mL \cdot kg^{-1} \cdot min^{-1}$)	61.1 ± 7.1	65.5 ± 5.6	54.9 ± 3.6	<0.001
$\dot{V}O2_{peak}$ ($mL \cdot kg\ FFM^{-1} \cdot min^{-1}$)	74.0 ± 5.1	75.2 ± 5.1	71.8 ± 4.7	0.171

Data are presented as mean ± SD. LT, power output at 4 mM lactate threshold; FFM, fat-free mass

Experimental design

Participants visited the laboratory for exercise tests on three separate occasions. All tests were performed on the same electronically-braked stationary cycle ergometer (Lode Excalibur Sport, Netherlands). Saddle height and handlebar position were adjusted to each participant's preference, but kept consistent for the three visits. Participants performed the exercise tests in their own cycling shoes with appropriate pedals provided by the laboratory.

Body mass and height were measured using digital scales (SECA, Germany) and a wall-mounted stadiometer (Holtain, UK), respectively. Body composition was measured using dual-energy x-ray absorptiometry (Lunar iDXA, GE Healthcare, UK).

During their first visit to the lab, participants performed a submaximal incremental exercise test to establish their lactate threshold, after which they performed an incremental exercise test to volitional exhaustion to establish their peak oxygen uptake ($\dot{V}O2_{peak}$). Two experimental trials, each comprised of a graded exercise test of six stages (at power outputs corresponding to approximately 30%, 40%, 50%, 60%, 70% and 80%$\dot{V}O2_{peak}$), with each stage lasting 8 min (Figure 3.1), were performed during subsequent visits in a randomized cross-over design. Each experimental trial was identical with the exception of a drink consumed in the hour prior to each exercise test, namely plain water (CON), or βHB salts (KET).

Incremental exercise tests

Assessment of lactate threshold and $\dot{V}O2_{peak}$ was performed in accordance guidelines from the British Association of Sport and Exercise Sciences (BASES) (Davison & Wooles, 2007; Spurway & Jones, 2007). Briefly, for determination of lactate threshold, participants completed 4 min stages (3 min of cycling and 1 min of rest), starting at 50 W. The power output was increased by 50 W for the next two stages, and 30 W thereafter until a blood lactate concentration (Lactate Pro 2, Japan) of 4 mM was exceeded. After a 15 min rest, $\dot{V}O2_{peak}$ was determined via an incremental test to exhaustion. Participants began cycling at a pre-determined power output based on body mass as per the BASES guidelines, and power output was progressively increased by was increased by 20 W.min-1 for males and 15 W.min-1 for females thereafter until volitional exhaustion.

Pre-trial preparation

All experimental trials were performed between 07:00 and 10:00, but on an individual basis, participants performed their second trial at the same time ±1 h as their first trial. Pre-trial preparation was the same for each experimental trial. Participants were asked to abstain from alcohol for 48 h and caffeine for at least 12 h, and refrain from strenuous exercise training for the day prior to each trial. Each trial took place after a standardized 10 h overnight fast. Participants were asked to keep a one-day portion estimate food diary for the day corresponding to two days prior to the first trial. They were instructed to repeat this pattern of intake before their second trial. On the day immediately prior to both experimental trials, participants were provided with a standardized diet (Gourmet Fuel, Ireland), which provided 40 kcal.kg body mass-1 at a macronutrient ratio of 40% carbohydrate, 30% protein and 30% fat. Male participants performed the two experimental trials separated by 7 or 14 days. Because the phase of the menstrual cycle influences fuel utilization during exercise (Oosthuyse & Bosch, 2010), female participants performed the two experimental trials separated by 7 days, but within the early to mid-luteal phase of their menstrual cycle.

Experimental trials

Experimental trials were performed in a randomized cross-over open-label design, and were identical with the exception of the drink consumed in the hour prior to each exercise test. The open-label design was chosen because of the difficulty in masking the pungent taste of the βHB salts, and considered acceptable because there was no performance element to the experimental design. Neither the study participants nor research personnel were blinded to allocation of condition, with the exception of the laboratory technician who did undertake analysis of the blood samples in a blinded manner.

During each trial, a bolus of a given drink was ingested at both 60 min and 15 min

prior to the commencement of exercise (Figure 3.1). Each bolus consisted of either (i) plain water provided at 3.8 mL.kg body mass-1 (CON), or (ii) βHB salts (KetoCaNa, Prototype Nutrition, IL USA) provided at 0.38 $g.kg^{-1}$ body mass dissolved in 3.8 mL.kg body mass-1 plain water (KET). Each bolus serving of KET provided ~18.5 g βHB, 2.1 g sodium and 1.8 g calcium, which is approximately 60% more βHB than the manufacturer's guidelines of 11.7 g βHB per serving. This timing and dosing strategy was based on our own pilot experiments (unpublished data) showing that plasma βHB concentration peaked at 60 min after ingestion of a single bolus, and that a greater elevation in plasma βHB concentration could be achieved with two smaller doses of βHB salts compared to a single larger dose equivalent to the same total amount of βHB salts.

Upon arrival at the laboratory, an indwelling catheter was introduced into an antecubital vein for serial blood sampling at rest (-60, -30 and 0 min) and during exercise (last 30 s of each 8 min stage) (Figure 3.1). The catheter was maintained patent between samples with saline (0.9% sodium chloride). The exercise test was graded and consisted of six stages at power outputs corresponding to approximately 30%, 40%, 50%, 60%, 70% and 80%$\dot{V}O2_{peak}$, with each stage lasting 8 min (Figure 3.1). Expired air was collected continuously throughout each exercise test on a breath-by-breath basis (COSMED Quark b2, Italy). During the last 30 s of each 8 min stage, HR (Polar, Finland) and RPE (Borg scale) were recorded, and a blood sample was collected for measurement of plasma βHB, lactate, and glucose concentrations.

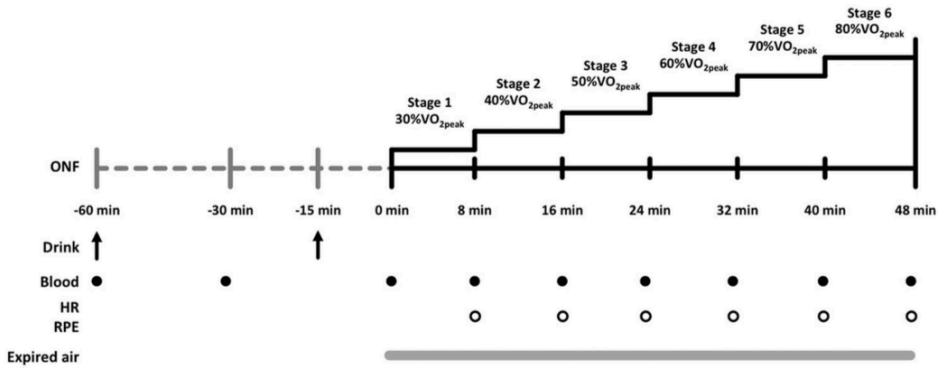

Figure 3.1 Experimental design schematic

Blood analysis

Blood samples (4 mL) were collected in plastic tubes containing sodium fluoride/potassium oxalate (Vacuette Glucose tubes, Greiner-Bio-One, Germany) for subsequent analysis. Samples were stored on ice before centrifugation at 3000 g for 10 min at 4C, after which three aliquots of plasma were separated for storage at -80C until later analysis of plasma βHB, lactate, and glucose (RX Daytona, Randox Laboratories, UK; assay codes RB1007, LC2389 and GL364 respectively).

Data analysis

Cardiopulmonary and metabolic parameters. Minute ventilation (VE), $\dot{V}O_2$, carbon dioxide production ($\dot{V}CO_2$) and RER were calculated from an average of breath-by-breath measurements during the last 30 s of each stage in the incremental exercise tests, and during the last 2 min of each stage in main experimental trials. Oxygen pulse (O_2 pulse), defined as oxygen uptake per heartbeat and expressed in $mL.beat^{-1}$, was calculated by dividing $\dot{V}O_2$ ($L.min^{-1}$) by HR ($beats.min^{-1}$) during the last 30 s of each stage.

Substrate utilization. The rate of energy expenditure ($kcal.min^{-1}$) during each stage was calculated from the average $\dot{V}O_2$ and $\dot{V}CO_2$ values during the last 30 s of each stage using equations applied on intensity-dependent basis (Jeukendrup & Wallis, 2005). Rates of

carbohydrate and fat oxidation are not reported because of the likely error introduced into these calculations by the oxidation of βHB and AcAc, which yield respiratory quotient values of 0.89 and 1.00 respectively (Frayn, 1983). Reporting oxidation rates based on RER is inaccurate during periods of nutritional ketosis unless appropriate correction factors for CO_2 displacement, and excretion of ketone bodies in urine and expired air are employed (Frayn, 1983), which were beyond the scope of the current work.

Mechanical efficiency. Gross efficiency (GE) was calculated as the ratio of the work performed per minute (W converted to $kJ.min^{-1}$) to the energy expended per minute ($kJ.min^{-1}$) at each stage, expressed as a percentage. Delta efficiency (DE) was calculated as the ratio of the change in work performed per minute to the change in energy expended per minute between each stage, expressed as a percentage (Gaesser & Brooks, 1975).

Statistical analysis

Data were evaluated using GraphPad Prism 6 (GraphPad Software, Inc., CA USA), and are presented as mean±SD, with the exception of Figure 3.2 where error bars represent 95% confidence intervals. The experiment was powered based on change in RER as the primary outcome, which was chosen as a measure of an altered metabolic response. Based on the aforementioned pilot data where a 0.034±0.015 difference in RER between KET and CON was observed, n=13 participants would have been required given $\alpha=0.05$ and $1-\beta=0.8$ (GPower v3.1). Independent samples t-tests were used to determine differences between male and female participants for baseline characteristics. Two-way (condition × intensity) repeated measures analysis of variance (ANOVA) was used to determine differences between the two experimental trials for variables with serial measurements. When a main effect of condition, or an interaction effect between condition and intensity, was indicated, post-hoc testing was performed using Holm-Sidak's multiple comparisons test with multiplicity-adjusted P values

to compare KET to CON at respective time points. The data were tested for normality and sphericity prior to proceeding with the tests described. In addition, standardised differences in the mean were used to assess magnitudes of effects between conditions at respective time points. These were calculated using Cohen's d effect size (ES) and interpreted using thresholds of <0.25, >0.25, >0.5 and >1.0 for trivial, small, moderate, and large, respectively (Rhea, 2004). Pearson's product-moment correlation coefficient (r) was used to explore correlations between variables. No differences were observed between male and female participants for the effect of KET on the metabolic response to exercise compared to water, so male and female data are presented as combined (n = 19) data unless otherwise stated. The significance level was set at $\alpha = 0.05$ for all statistical tests.

Results

Plasma βHB, glucose and lactate. Fasting plasma βHB (KET, 0.13±0.10 mM; CON, 0.12±0.09 mM; ES=.05) and glucose (KET, 4.82±0.46 mM; CON, 4.79 ±0.40 mM; ES=.06) concentrations did not differ between trials (Figure 3.2). Ingestion of KET resulted in a rise in plasma βHB concentration to 0.28±0.13 mM (P<.001) 30 min after ingestion, and remained elevated throughout exercise (P<.001) (Figure 3.2a). The highest plasma βHB concentration during KET was observed in the final stage of exercise at 0.44±0.15 mM (P<.001). Plasma glucose concentration averaged 0.44±0.27 mM lower 30 min after ingestion of KET compared to CON (P=.008; ES=.96). An inverse correlation (r=-0.647, P=.004) was observed for the change in plasma βHB and glucose concentrations at this time point. Plasma glucose concentrations remained lower at all stages throughout exercise, with effect sizes indicating small to moderate effects i.e. 30%$\dot{V}O_{2peak}$, -0.19±0.36 mM, ES=.39; 40%,$\dot{V}O_{2peak}$, -0.21±0.43 mM, ES=.44; 50%$\dot{V}O_{2peak}$, -0.27±0.40 mM, ES=.66; 60%$\dot{V}O_{2peak}$, -0.21±0.39 mM, ES=.62;

70%$\dot{V}O_{2peak}$, -0.17±0.54 mM, ES=.33; and 80%$\dot{V}O_{2peak}$, -0.39±1.24 mM, ES=.34 (Figure 3.2b). Plasma lactate concentrations were elevated above resting values during the final two stages of exercise, but no differences between KET and CON were observed for plasma lactate concentrations at any time point (Figure 3.2c).

Cardiorespiratory responses to graded exercise and KET ingestion. All cardiorespiratory parameters exhibited main effects for exercise intensity (all P<.001). No differences in %$\dot{V}O_{2peak}$, $\dot{V}O_2$, $\dot{V}CO_2$, or VE were observed between conditions (Table 3.2). RER was elevated by KET (P<.001 for condition), and was ~0.03 higher for intensities up to 60%$\dot{V}O_{2peak}$ (all P<.05), with effect sizes indicating moderate effects at these intensities i.e. 30%$\dot{V}O_{2peak}$, 0.038±0.030, P=0.003, ES=.90; 40%,$\dot{V}O_{2peak}$, 0.035±0.036, P=0.007, ES=.92; 50%$\dot{V}O_{2peak}$, 0.028±0.031, P=0.025, ES=.81; and 60%$\dot{V}O_{2peak}$, 0.027±0.037, P=0.031, ES=.78 (Figure 3.2d). The effect of KET on RER was small at 70%$\dot{V}O_{2peak}$ (0.018±0.030, P=0.16, ES=.50) and 80%$\dot{V}O_{2peak}$ (0.012±0.045, P=0.37, ES=.28). HR was also elevated by KET (P=.003 for condition), wherein HR averaged ~4 to 8 bpm higher during KET and effect sizes indicated small to moderate effects i.e. 30%$\dot{V}O_{2peak}$, 5.6±4.5 bpm, ES=.48; 40% $\dot{V}O_{2peak}$, 8.5±7.1 bpm, ES=.66; 50%$\dot{V}O_{2peak}$, 7.8±7.1 bpm, ES=.55; 60%$\dot{V}O_{2peak}$, 3.9±8.6, ES=.26; 70%$\dot{V}O_{2peak}$, 4.9±8.2 bpm, ES=.29; and 80%$\dot{V}O_{2peak}$, 4.4±7.0 bpm, ES=.34 (Figure 3.2e). No differences in oxygen pulse, RPE, gross efficiency or delta efficiency were observed between conditions (Table 3.2).

Gastrointestinal responses. Thirteen out of nineteen (68%) participants reported symptoms of gastrointestinal distress in response to KET ingestion. These comprised of seven (37%), three (16%), two (11%), and one (5%) of the participants reporting nausea, diarrhoea, vomiting and light-headedness, respectively. These symptoms manifested in the latter stages of and immediately after the cessation of exercise. No symptoms were reported during CON

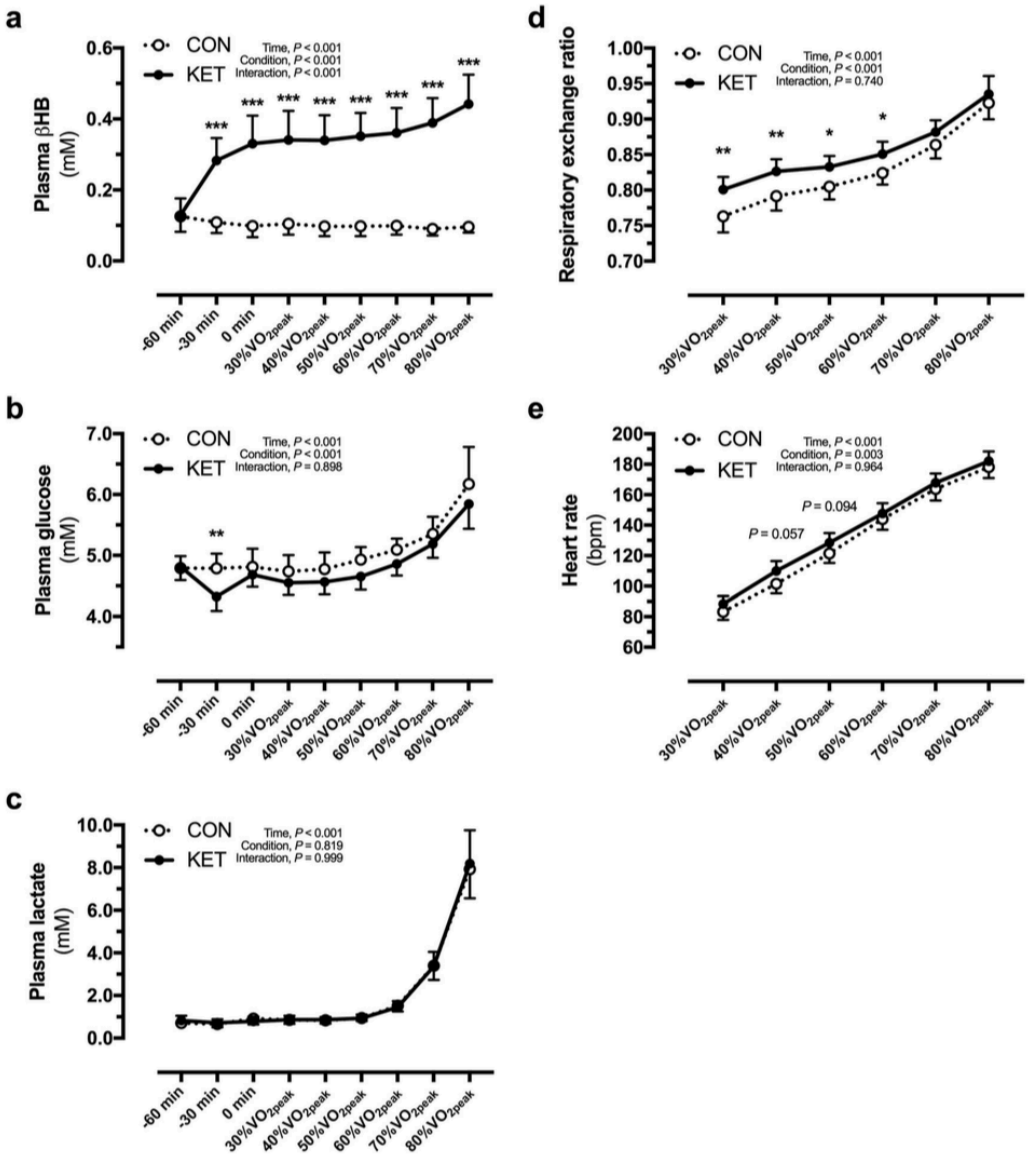

Figure 3.2 Plasma βHB (a), glucose (b) and lactate (c), respiratory exchange ratio (d), and HR (e) responses after βHB salt ingestion and water ingestion.

Data are presented as mean values, with error bars representing 95% confidence intervals. *p < .05 KET vs. CON; ** p < .01 KET vs. CON; ***p < .001 KET vs. CON; KET, βHB salt ingestion; CON, water ingestion; HR, heart rate; RER, respiratory exchange ratio.

Table 3.2 Cardiorespiratory responses during graded exercise in CON or KET.

	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Stage 6
%W_{max}						
KET	16±5	28±4	40±4	52±4	64±4	75±4
CON	16±5	28±4	40±4	52±4	64±4	75±4
%$\dot{V}O_{2peak}$						
KET	29±3	39±2	50±3	61±3	74±4	85±4
CON	29±3	39±2	50±2	61±3	74±4	86±5
VE ($L \cdot min^{-1}$)						
KET	30.7±5.5	40.8±5.3	52.6±5.3	66.2±7.7	89.9±10.8	127.6±16.1
CON	30.0±4.6	40.0±5.0	52.2±5.0	66.3±6.2	89.1±9.6	124.5±23.5
$\dot{V}O_2$ ($L \cdot min^{-1}$)						
KET	1.42±216	1.89±151	2.42±152	2.96±210	3.55±257	4.09±299

CON	1.40±183	1.87±201	2.41±206	2.96±270	3.58±322	4.11±444
$\dot{V}CO_2$ ($L \cdot min^{-1}$)						
KET	1.15±195	1.57±172	2.04±183	2.53±243	3.16±293	3.90±360
CON	1.09±163	1.49±175	1.95±179	2.44±222	3.10±305	3.80±424
RPE						
KET	7±1	9±1	11±1	13±2	15±1	18±2
CON	6±1	8±1	11±1	13±1	15±1	17±2
O_2 pulse ($mL \cdot beat^{-1}$)						
KET	13±3	15±3	16±3	17±3	19±4	19±4
CON	14±4	16±4	18±4	18±4	19±4	21±5

Data are presented as mean ± SD. %Wmax, percentage of maximum power output; %$\dot{V}O_2$peak, percentage or peak oxygen uptake; VE, minute ventilation; $\dot{V}O_2$, rate of oxygen uptake; $\dot{V}CO_2$, rate of carbon dioxide production; RPE, rating of perceived exertion.

Discussion

The aim of the present study was to investigate the effect, if any, of acute ingestion of βHB salts on metabolic and physiological responses to a graded exercise session in trained cyclists. Ingestion of commercially-available βHB salts resulted in elevated plasma βHB concentrations (>0.3 mM) at rest and during exercise. This coincided with elevated RER (moderate effects) and HR (small to moderate effects) during submaximal exercise intensities, and a lowering of plasma glucose concentrations (small to moderate effects), compared to the ingestion of water. However, a range of other parameters including plasma lactate, rating of perceived exertion, gross efficiency, and delta efficiency were unaffected by the acute ingestion of βHB salts.

Exogenous ketone supplements, such as βHB salts, represent a novel method to increase the concentration of circulating ketone bodies without implementing restrictive dietary practices such as fasting or low carbohydrate, ketogenic diets (Cox & Clarke, 2014; Evans et al., 2017). Despite the increasing commercial availability of βHB salts, to date there is a paucity of data from humans on the metabolic response to ingestion at rest or during exercise. The sodium/potassium βHB mineral salt ingested in the present study resulted in a modest elevation (~0.3 to 0.4 mM) in plasma βHB concentrations. These values are similar to those observed after a 24 h fast (Balasse & Fery, 1989; Laffel, 1999), and can be considered to have produced nutritional ketosis (i.e. >0.2 mM (Robinson & Williamson, 1980). The dosing strategy employed involved a bolus ingested both at 60 min and 15 min prior to exercise, but plasma βHB concentrations peaked during the last stage of exercise at 0.44±0.15 mM. This suggests that the supplement was still being released into circulation approximately one hour after the ingestion of the second bolus, a time course consistent with several recent reports describing βHB salt ingestion at rest (Stubbs et al., 2017), and prior to exercise (O'Malley et al., 2017; Rodger et al., 2017). However, these studies reported

somewhat higher blood βHB concentrations (~0.6 to 1.0 mM) after ingestion at doses providing of two boluses of 11.7 g of βHB (Rodger et al., 2017), ~21 to 27 g of βHB (O'Malley et al., 2017), and ~12 g or ~25 g of βHB (Stubbs et al., 2017), compared to the two doses of ~18.5 g in the present study. However, unlike the present study, these studies measured βHB concentrations in whole blood from finger-prick sampling using handheld monitors, which are known to over-estimate blood βHB concentration ranging from 50% to three-fold relative to lab-based measures performed on serum (Guimont et al., 2015; Leckey et al., 2017).

The aim of ingestion of exogenous ketone supplements is to achieve acute nutritional ketosis (Cox & Clarke, 2014), and this is readily-achieved by the (R)-3-hydroxybutyl (R)-3-hydroxybutyrate ketone monoester (Cox et al., 2016). Ingestion of 573 $mg.kg^{-1}$ body mass of that supplement raises plasma βHB concentrations to ~3 mM 10 min after ingestion, which rise further to ~6 mM within the next 60 min at rest (Cox et al., 2016). Clearly the βHB salts ingested in the present study produce plasma βHB concentrations that are ~10-fold less than this. Despite the modest change in plasma βHB concentrations, the acute ingestion of βHB salts does exert some metabolic action at rest and during exercise. For instance, a ~10% decline in plasma glucose was observed 30 min after the ingestion of βHB salts, with an inverse correlation observed between the respective changes in plasma βHB and glucose concentrations at this time. This is consistent with the acute infusion of ketone bodies producing βHB concentrations of ~0.5 to 1 mM resulting in a decline in plasma glucose of ~10% (Mikkelsen et al., 2015; Sherwin et al., 1975), and similar results associated with βHB salt ingestion (Stubbs et al., 2017). Moreover, a slightly lower plasma glucose concentration (~0.2 mM; small to moderate effects) was evident throughout exercise in the present study, which confirms other recent reports (Leckey et al., 2017; O'Malley et al., 2017; Rodger et al., 2017).

Other effects observed during exercise in the present study include elevations in RER (moderate effects) and HR (small to moderate effects) during the low-to-moderate intensities of exercise. The elevation in RER may be indicative of oxidation of ketone bodies during exercise based on the stoichiometry of oxidation of AcAc. Before being oxidized as a fuel source in skeletal muscle, βHB is re-oxidized to AcAc through the action of 3-hydroxybutyrate dehydrogenase (BDH). The respiratory quotient for oxidation of AcAc is similar to that glucose at a value of 1.0 (Frayn, 1983). Therefore, a contribution of ketone oxidation to energy provision likely explains the elevation in RER during exercise after ingestion of βHB salts in the present study. The elevated RER is consistent with a recent report of prolonged submaximal exercise in trained male cyclists (Rodger et al., 2017), but the opposite of what was reported during graded exercise in recreationally-active men (O'Malley et al., 2017). Like the former study, we studied trained cyclists, so whether training status is the only explanation for the divergent findings remains to be confirmed. However, this would be consistent with our previous suggestion that oxidation of ketone bodies during exercise is likely to be greatest in trained participants with a high proportion of type I muscle fibres and/or a high oxidative capacity in skeletal muscle (Evans et al., 2017).

Calculations of arteriovenous differences of radiolabelled ketone bodies across working muscles estimate the contribution of ketone bodies to energy provision of 2% to 10% during exercise in the fasted state (Balasse et al., 1978; Fery & Balasse, 1983; Wahren et al., 1984). This contribution is unlikely to be >10% unless plasma βHB concentrations are elevated above 1 mM and exercise is being performed by trained participants (Evans et al., 2017). In well-trained participants consuming exogenous ketones as a ketone monoester, the contribution of ketone bodies to energy provision is greater i.e. 16 to 18% of total oxygen consumption (Cox et al., 2016). Therefore, although the elevation in plasma βHB concentration in the present study was modest, it is likely that this did result in an increased

contribution of ketone bodies to energy provision during exercise.

Apart from a contribution to energy provision, the principal efficacy of supplemental ketones as a performance aid is likely to be secondary effects on metabolism and alterations in fuel selection (Evans et al., 2017). For instance, acute infusion of sodium AcAc (Fery & Balasse, 1988) or sodium βHB (Lestan et al., 1994) attenuates the exercise-induced rise in plasma lactate, an effect also observed after ingestion of the aforementioned ketone monoester (Cox et al., 2016). In the latter work, reduced glycolytic flux, glycogen sparing, and increased contribution of intramuscular triglyceride to energy provision were observed during 2 h of cycling at ~70%$\dot{V}O_{2peak}$. However, an attenuation of the rise in plasma lactate was not observed in the present study, or in other recent studies examining acute ingestion of βHB salts (O'Malley et al., 2017; Rodger et al., 2017). Again, this might be explained by the relatively lower increase in plasma βHB concentration produced by the βHB salts compared to the ketone monoester.

An important methodological note is that the βHB salts used in the present study provide a racemic mixture of βHB i.e. containing both the D- and L- enantiomers of βHB (also designated R- and S- respectively), whereas the βHB assay employed determines the concentration of D-βHB. D-βHB is the circulating and primary form of βHB (Tsai et al., 2006), but intracellular concentrations of L-βHB are sensitive to factors such as aging and metabolic health (Hsu et al., 2011). The D- and L- enantiomers of βHB exert divergent physiological effects on glucose metabolism in the heart (Tsai et al., 2006) and skeletal muscle (Yamada, Zhang, Westerblad, & Katz, 2010), and on longevity (Edwards et al., 2014). Recent work has demonstrated that racemic βHB ingested as βHB salts results in elevations in L-βHB concentrations of ~2 mM (Stubbs et al., 2017). However, it is doubtful that a change in circulating L-βHB concentration, if provided by an exogenous ketone supplement,

would have any direct effect on substrate metabolism in skeletal muscle. For instance, L-βHB is not a substrate for mitochondrial BDH and thus is not metabolized to AcAc (Scofield et al., 1982), and its physiological role is most likely in the synthesis of sterols and fatty acids in non-muscle tissues (Webber & Edmond, 1977).

The small to moderate effects observed for an elevated heart rate of 4 to 8 bpm after ingestion of βHB salts compared to water may warrant future investigation. Heart rate during exercise was not reported in previous work with βHB infusion, or ketone monoester or βHB salt ingestion, but was elevated by 25% under resting conditions after sodium βHB infusion compared to saline infusion (Gormsen et al., 2017). This indicates an effect of βHB itself rather than sodium load, but occurred at a plasma βHB concentration of ~4 mM in contrast to ~0.4 mM in the present study. Alternatively, the sodium load delivered by the βHB salts may exert some hemodynamic effects. Acute sodium ingestion can transiently elevate blood pressure (Farquhar et al., 2005), and sodium bicarbonate ingestion providing a similar dose of sodium to the present study results in an elevation in heart rate of ~10 bpm during moderate intensity exercise (Kahle et al., 2013).

Also notable in the present study was that thirteen out of nineteen (68%) participants reported symptoms of gastrointestinal distress after exercise in the βHB salt condition. The hypertonic nature of the βHB salts ingested likely caused an intraluminal osmotic load and water shift into the intestinal lumen resulting in osmotic diarrhoea. However, gastrointestinal distress also is a potential side effect of acute ingestion of ketone esters, with high prevalence noted after the ingestion of the ketone diester by elite cyclists (Leckey et al., 2017), and increasing incidences occurring with increasing dosages of the ketone monoester, however, this may be confounded by the increase in litres per day of the milk based meal replacement shake (Clarke et al., 2012). Clearly such issues would be deleterious to exercise performance,

and therefore require further exploration, either in terms of optimal dosing strategies, or whether repeat exposure to exogenous ketone supplements reduces these symptoms.

In conclusion, acute ingestion of a commercially-available βHB salt formulation by trained cyclists resulted in a modest increase in plasma βHB concentrations before and during graded exercise to concentrations that can be considered acute nutritional ketosis. This resulted in alterations in the metabolic and physiological response to exercise as evidenced by lowering of plasma glucose concentrations, and elevated RER and heart rate values at low-to-moderate exercise intensities compared to ingestion of water. However, no effect was observed on perceived exertion or muscular efficiency, or on plasma lactate concentrations. This is in contrast to previous work using βHB infusion or ingestion of a ketone monoester supplement, both of which achieve markedly higher plasma βHB concentrations during exercise. This suggests the likelihood that a dose-response effect exists for exogenous ketone supplements on metabolic responses and exercise performance. Given the gastrointestinal issues observed with the present βHB salts, further work is needed with other methods of increasing circulating ketone concentrations including improved free acid or mineral salt formulations, before the merit, if any, of ketone salts for performance enhancement in athletes is likely to be realised.

Chapter 4

Evans M, Egan B (2018). Intermittent running and cognitive performance after ketone ester ingestion. *Medicine & Science in Sports & Exercise* 50(11):2330-2338.

Abstract

Introduction: Ingestion of exogenous ketones alters the metabolic response to exercise and may improve exercise performance, but has not been explored in variable intensity team sport activity, or for effects on cognitive function. **Methods:** On two occasions in a double-blind, randomised crossover design, eleven male team sport athletes performed the Loughborough Intermittent Shuttle Test (Part A, 5x15 min intermittent running; Part B, shuttle run to exhaustion), with a cognitive test battery before and after. A 6.4% carbohydrate-electrolyte solution was consumed before and during exercise either alone (PLA), or with 750 $mg.kg^{-1}$ of a ketone ester supplement (KE). Heart rate (HR), rating of perceived exertion (RPE), and 15 m sprint times were recorded throughout, and serial venous blood samples were assayed for plasma glucose, lactate and β- hydroxybutyrate (βHB). **Results:** KE resulted in plasma βHB concentrations of ~1.5 to 2.6 mM during exercise ($P<0.001$). Plasma glucose and lactate concentrations were lower during KE compared to PLA (moderate-to-large effect sizes). HR, RPE and 15 m sprint times did not differ between trials. Run time to exhaustion was not different ($P=0.126$, $d=0.45$) between PLA [(mean (95% CI); 268, (199, 336) sec) and KE (229, (178, 280) sec). Incorrect responses in a multi- tasking test increased from pre- to post-exercise in PLA [1.8 (-0.6, 4.1)] but not KE [0.0 (-1.8, 1.8)] ($P=0.017$; $d=0.70$). **Discussion:** Compared to carbohydrate alone, co-ingestion of a ketone ester by team sport athletes attenuated the rise in plasma lactate concentrations, but did not improve shuttle run time to exhaustion or 15 m sprint times during intermittent running. An attenuation of the decline in executive function after exhausting exercise suggests a cognitive benefit after KE ingestion.

Introduction

Ketone bodies, namely β-hydroxybutyrate (βHB), acetoacetate (AcAc) and acetone, are fatty acid metabolites whose production markedly increases in physiological states characterised by reduced glucose availability, such as starvation and ketogenic diets (Robinson and Williamson, 1980; Balasse & Fery, 1989). Ketone bodies are principally produced as a survival mechanism to provide a substrate for the brain, but are also oxidised by skeletal muscle and provide up to 10% of energy during exercise in a fasted state (Fery & Balasse, 1986). Infusion of ketone bodies exerts a range of metabolic actions, such as attenuation of hepatic glucose output, anti-lipolytic effects in adipose tissue, and in skeletal muscle, glucose 'sparing' and stimulation of protein synthesis (Maizels et al., 1977; Nair et al., 1988; Mikkelsen et al., 2015).

The effects of ketone bodies on substrate utilisation during exercise, and consequently athletic performance, is of increasing interest due to the development of exogenous ketone supplements, namely ketone salts and ketone esters (Clarke et al., 2012; Kesl et al., 2016; Stubbs et al., 2017). These formulations represent a method of acutely inducing nutritional ketosis (plasma βHB >0.5 mM) resulting in a variety of effects on exercise metabolism, performance and recovery (Cox et al., 2016; Holdsworth et al., 2017; O'Malley et al., 2017; Vandoorne et al., 2017; Rodger et al., 2017; Leckey et al., 2017; Evans et al., 2018; Waldman et al., 2018). Ketone salts in their presently-available racemic form produce only modest changes (<1.0 mM) in plasma βHB concentrations (Stubbs et al., 2017; O'Malley et al., 2017; Rodger et al., 2017; Evans et al., 2018; Waldman et al., 2018). While their pre-exercise ingestion can alter the metabolic response to exercise (O'Malley et al., 2017; Evans et al., 2018), there is no evidence of an ergogenic effect (O'Malley et al., 2017; Rodger et al., 2017). Alternatively, exogenous ketone supplements in the form of ketone esters produce markedly

greater changes in plasma βHB concentrations than ketone salts in humans (Stubbs et al., 2017) and rats (Kesl et al., 2016).

Two ketone esters have been reported in the recent literature: a R,S-1,3-butanediol acetoacetate diester (KDE) (Kesl et al., 2016; Leckey et al., 2017) and a (R)-3-hydroxybutyl (R)-3-hydroxybutyrate ketone monoester (KME) (Clarke et al., 2012; Stubbs et al., 2017; Cox et al., 2016). Both esters were tested in elite endurance athletes with divergent findings (Cox et al., 2016; Leckey et al., 2017). Acute ingestion of KME produced plasma βHB concentrations of ~3.0 mM after 20 min, and improved 30 min time-trial performance by 2% (Cox et al., 2016)). In contrast, acute ingestion of KDE was less effective at raising serum βHB concentrations (~0.4 mM) and impaired 31.2 km time-trial performance by 2% (Leckey et al., 2017). Consumption of KME increased the estimated contribution of ketone bodies to fuel provision during exercise to 16-18% of total energy provision, in addition to marked metabolic effects including the attenuation of blood lactate concentrations, 'sparing' of muscle glycogen, and increased intramuscular triglyceride utilisation (Cox et al., 2016). The reduction in glycolytic flux may reflect an impairment of glycogen utilisation rather than glycogen sparing (Burke, 2015; Pinckaers et al., 2017; Evans, Cogan and Egan, 2017), a key question that requires further investigation. The former is likely to impair performance in high intensity sports that demand a high rate of ATP provision from carbohydrate sources (Burke, 2015).

Team sports such as Australian football, soccer, Gaelic games, rugby union, lacrosse and field hockey are high-intensity and intermittent in nature, consisting of repeated periods of high intensity activity (sprinting) interspersed with exercise at low-to-moderate intensities (walking, jogging) (Spencer et al., 2005; Cummins et al., 2013). Nutrition guidelines for soccer, for instance, recommend high intakes of carbohydrate prior to and during competition to maximise muscle glycogen stores with the view to enhancing performance (Burke et al.,

2006). Soccer match play results in a marked reduction in muscle glycogen, and high intensity running is attenuated in the last 15 minutes of play (Jacobs et al., 1982; Mohr et al., 2003). Therefore, nutrition strategies that could spare glycogen and maintain high intensity running in the latter parts of matches are of interest to scientists and practitioners, but research on exogenous ketone supplements to date has mostly focused on athletes from endurance sports (Cox et al., 2016; Rodger et al., 2017; Leckey et al., 2017; Evans et al., 2017). Moreover, because ketone bodies are the dominant fuel source for the brain in ketogenic states (Owen et al., 1967), there is potential for central and/or cognitive effects of exogenous ketone supplements, but to date cognitive effects were only explored in short-term feeding trials in rats (Ari et al., 2016; Kovacs et al., 2017; Murray et al., 2016), Therefore, the aim of the present study was to investigate the effects of acute ingestion of a ketone ester on metabolic responses, physical and cognitive performance in team sport athletes in response to an intermittent running protocol that simulated soccer match play. We hypothesise that ingestion of a ketone ester will have no effect on physical or cognitive performance in response to the intermittent running protocol.

Methods

Participants

Eleven male team sport athletes (mean±SD: age, 25.4±4.6 y; height 1.80±0.05 cm; body mass, 78.6±5.3 kg; $\dot{V}O_{2peak}$ 53.9±2.2 $mL \cdot kg^{-1} \cdot min^{-1}$) gave written informed consent to participate after written and verbal explanation of the procedures. Ethical approval (permit number: DCUREC2017_130) was obtained from the Dublin City University Research Ethics Committee in accordance with the Declaration of Helsinki. All participants were actively training and competing in high-intensity field-based team sports.

Experimental design

Participants visited the laboratory for exercise tests on three separate occasions over a 14 to 21 day period. During their first visit to the lab, each participant's maximal oxygen consumption ($\dot{V}O_{2peak}$) and speed at $\dot{V}O_{2peak}$ were determined using a progressive multistage shuttle run test (Yo-Yo intermittent recovery test level 1; Yo-Yo IR1) (Bangsbo et al., 2008). These data were used to determine jogging (55% $\dot{V}O_{2peak}$) and cruising (95% $\dot{V}O_{2peak}$) speeds for use during the Loughborough Intermittent Shuttle Test (LIST). The LIST is a validated simulation of the physiological and metabolic responses during soccer match play and consists of two parts: Part A comprises a fixed period of variable intensity shuttle running over 20 m; Part B consists of continuous running, alternating every 20 m between 55% and 95% $\dot{V}O_{2peak}$ until volitional fatigue (Nicholas et al., 2000). After a 15 min rest after completion of the Yo-Yo IR1, participants were familiarised with the LIST protocol by completing one 15 min block at their personalised running speeds. Cognitive tests were performed before the Yo-Yo IR1 and after familiarisation with the LIST in order to familiarise participants with the cognitive test battery.

Two main experimental trials, each comprising of the LIST (Parts A and B) with cognitive tests before and after, were performed during subsequent visits in a double-blinded, randomised cross-over design. Both experimental trials included a standardised diet for ~36 h prior to the exercise test, and were identical except for the drinks consumed before and during the LIST, namely a 6.4% carbohydrate-electrolyte solution, which was either flavoured (Symrise, UK) and acted as the control/placebo condition (PLA), or included a ketone ester (KE) (Figure 3.1). The primary outcome was endurance capacity measured by run time to exhaustion in the LIST Part B, with secondary outcomes including 15 m sprint times during the LIST Part A, heart rate (HR), rating of perceived exertion (RPE) and plasma glucose, lactate and βHB concentrations.

Incremental exercise test and familiarisation

For determination of $\dot{V}O_{2peak}$, jogging (55% $\dot{V}O_{2peak}$) and cruising (95% $\dot{V}O_{2peak}$) during the LIST, participants completed the Yo-Yo IR1. All participants completed a standardised 5 min warm-up consisting of progressive shuttle runs at 20%, 40%, 60% and 80% $\dot{V}O_{2peak}$ and dynamic stretching (high knees, heel kicks, groin bridges), followed by a period of self-selected stretching. The Yo-Yo IR1 consists of 40 m shuttle runs (2x20 m) between two sets of cones set 20 m apart. Shuttles progressively increase in speed that is dictated by an audio signal (Teambeep Software, UK). Each 40 m shuttle is separated by a 10 s rest period. The test was terminated when participants failed to complete the second 20 m shuttle on two consecutive occasions or if they reached volitional fatigue. $\dot{V}O_{2peak}$ was calculated as

$\dot{V}O_{2peak}$ ($mL \cdot kg^{-1} \cdot min^{-1}$) = Yo-Yo IR1 distance (m) × 0.0084 + 36.4.

After a 15 min break, participants were familiarised with the LIST by performing one block of intermittent activity i.e. 15 min of Part A, were allowed 3 min of rest, and then completed the Part B run to exhaustion. Participants completed a battery of cognitive tests before the Yo-Yo IR1 and after the intermittent run to fatigue.

Cognitive test battery

The battery of cognitive tests (CANTAB Cognition, UK) was administered via a touch screen tablet lasting ~25 min. An identical test battery was administered before and after each trial. Technical issues, namely with loss of wireless internet access during test administration, resulted in the data for the cognitive test battery comprising of n=8 participants.

During the reaction time (RTI) task, participants select and hold a button at the bottom of the screen and five circles are presented above. In each case, a yellow dot appears in one of the five circles, and the participant must react as soon as possible, releasing the

button at the bottom of the screen, and selecting the circle in which the dot appeared. Release time (msec), reaction time (msec), and number of errors were recorded.

The multi-tasking test (MTT) is a test of executive function that measures the participant's ability to switch attention between stimuli, and ignore task-irrelevant information. White arrows are displayed on a black background, with the arrows located on either the left or right side of the screen, and pointing either to the left or to the right. A cue is displayed at the same time as the arrows, reading either "SIDE" or "DIRECTION." When the "SIDE" cue is presented, the participant is required to press a button on the left or right of the screen corresponding to the side of the screen where the arrow is presented, regardless of the direction the arrow is pointing. Conversely, when the "DIRECTION" cue is presented, the participant is required to touch a button on the left or right of the screen corresponding to the direction the arrow is pointing, regardless of which side of the screen the arrow is presented. Reaction time (msec), and number of correct and incorrect responses were recorded.

The rapid visual information processing task (RVP) is a test of sustained attention. The participant is presented with a white box in the center of the screen. Single digits ranging from 2 to 9 are presented one at a time in a pseudo-random order inside the box, appearing at a rate of 100 digits per minute. The participant is required to detect specific 3-digit sequences, including 2-4-6, 4-6-8, and 3-5-7. As soon as a target sequence is detected, the participant is required to touch a button on the screen. Response latency (msec), correct responses and false alarms were recorded.

Pre-trial preparation

All experimental trials were performed between 15:30 and 20:00, but on an individual basis, participants performed their second trial at the same time ±1 h as their first trial. Pre-trial preparation was the same for each experimental trial. Participants were asked to abstain from alcohol for 48 h and caffeine for 24 h, and refrain from strenuous exercise training the

day prior to each trial. The day prior to experimental trials, participants were provided with a standardised diet (Gourmet Fuel, Ireland), which provided 40 kcal·kg body mass^{-1} at a macronutrient ratio of 60% carbohydrate, 20% protein and 20% fat. On the day of experimental trials, participants consumed two meals providing 3 g·kg body mass^{-1} of carbohydrate before arriving at the lab. The second meal was consumed 3 h before the initiation of the LIST. In addition to the energy content and macronutrient ratio, the food itself was identical for both trials. Participants performed the two experimental trials separated by either 7 or 14 days.

Experimental trials

Experimental trials were performed in a double-blinded, randomised cross-over design, and were identical except for the drinks consumed. During each trial, a bolus of a given drink was ingested 20 min prior to exercise (drink 1), and during each 3 min seated break during Part A (drinks 2 to 6) (Figure 4.1). During PLA, a 6.4% carbohydrate-electrolyte solution (Lucozade Sport, Lucozade Ribena Suntory Ltd., UK) was provided at a rate of ~1.2 g·min^{-1} of exercise. During KE, a 6.4% carbohydrate-electrolyte solution was provided at a rate of ~1.2 g·min^{-1} combined with 750 mg·kg^{-1} body mass of a R-βHB (R)1,3-butanediol ketone ester (KE4, KetoneAid, USA). The ketone ester was mixed directly with the carbohydrate-electrolyte solution for ingestion in three boluses (50:25:25) i.e. at 20 min prior to exercise (drink 1), and after 30 (drink 3) and 60 min (drink 5) of exercise, respectively (Figure 4.1). During PLA, drinks 1, 3 and 5, were flavoured with a bitter additive (Symrise, UK) to taste-match with KE, and in both trials, drinks 2, 4, and 6 were provided as the unadulterated carbohydrate-electrolyte solution. All drinks were administered in opaque drinks bottles.

Upon arrival at the laboratory, participants provided a urine sample for assessment of hydration status (PalOSMO, VITECH Scientific, Japan), and then proceeded to complete the

described battery of cognitive tests. Thereafter, an indwelling catheter (21G Insyte Autoguard; Becton Dickinson, USA) was introduced into an antecubital vein for serial venous blood sampling at rest (-20 and 0 min), during each 3 min seated rest period between the 15 min blocks in Part A, and immediately after the run to exhaustion. Participants were fitted with a Bluetooth heart rate monitor (Polar V7, Polar Electro Oy, Finland) for continuous recording of HR, and then performed the standardised 10 min warm up followed by self-selected stretching. Participants then performed the LIST protocol (Part A: 5x15 min intermittent activity; Part B: run to exhaustion) (Nicholas, Nuttall and Williams, 2000). All exercise intensities were based on percentages of $\dot{V}O_{2peak}$ determined during the Yo-Yo IR1. The repeating order of activity in Part A, which occurs in a continuous manner for each 15 min block, comprises of 3 x 20 m at walking speed, 1 x maximal 15 m sprint, 4 sec recovery, 3 x 20 m jogging speed (55% $\dot{V}O_{2peak}$) and 3 x 20 m at cruising speed (95% $\dot{V}O_{2peak}$). Sprint times were measured by two sets of wireless infrared photoelectric cells (TC Timing System; Brower Timing, USA).

Part B consists of single 20 m shuttles alternating between the jogging (55% $\dot{V}O_{2peak}$) and cruising (95% $\dot{V}O_{2peak}$) speeds. The shuttle run to exhaustion, measured in sec, continued until participants were unable to complete two consecutive shuttles at cruising speeds, or until volitional fatigue. All speeds were dictated using audio software (Team Beep Software, UK). All participants received consistent encouragement during the maximal sprinting of Part A, and the run to exhaustion of Part B.

Venous blood samples were collected during the 3 min break between each 15 min block of Part A, and RPE (Borg scale) was recorded during the same time period. Incidences of gastrointestinal (GI) symptoms were recorded by interview after each trial after completion of the cognitive test battery. After completion of both experimental trials participants

completed an exit interview in which they were asked whether they could identify the KE condition, and which trial did they believe that they performed their longest run to exhaustion.

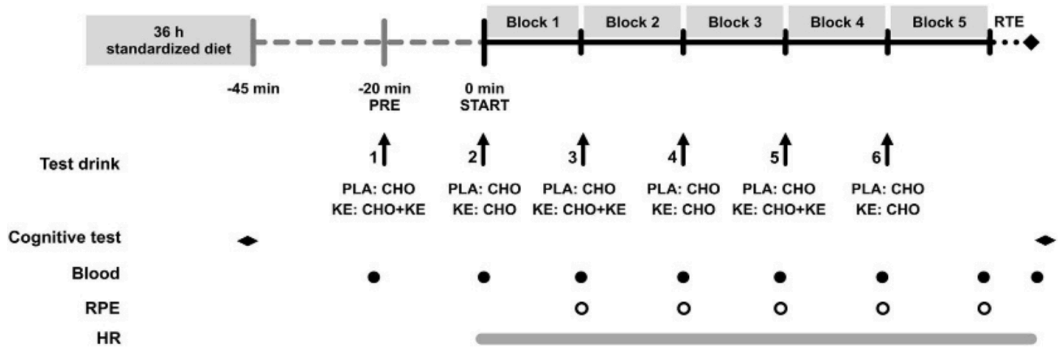

Figure 4.1 Schematic of the study protocol. CHO, carbohydrate-electrolyte solution; PLA, placebo; RTE, shuttle run to exhaustion

Blood analysis

Blood was collected in plastic tubes (2 mL) containing sodium heparin (Plus Blood Collection Tubes; Becton Dickinson, USA) for subsequent analysis of βHB). A second blood sample was collected in plastic tubes (4 mL) containing sodium fluoride (Plus Blood Collection Tubes; Becton Dickinson, USA), Samples were stored on ice before centrifugation at 3000 *g* for 10 min at 4 C, after which three aliquots of plasma were separated for storage at -80°C until later analysis of plasma βHB, lactate and glucose (RX Daytona, Randox Laboratories, UK: assay codes RB1007, LC2389 and GL364 respectively).

Statistical analysis

Data were evaluated using Prism 7.0 (GraphPad Software, Inc., CA, USA) and are presented as mean (lower, upper 95% confidence interval of the mean), with the exception of the participant characteristics, which are described as mean±SD. A paired samples t-test was used to determine differences between trials in run time to exhaustion and average HR during Part B. Two-way (time x condition) repeated measures analysis of variance (ANOVA) was used to determine differences between the two experimental trials for all other with variables

with serial measurements. When a main effect of condition, or an interaction effect between condition and time was indicated, *post-hoc* testing was performed with Bonferroni's correction with multiplicity-adjusted *P* values applied to compare KE to PLA at the respective time points. The data were tested for normality using the Shapiro-Wilk test prior to proceeding with the parametric tests described. For null hypothesis statistical testing, the significance level was set at $\alpha = 0.05$ for all tests. Apart from and independent of the outcome of the repeated measures ANOVA, standardized differences in the mean were used to assess magnitudes of effects between conditions at respective time points. These effect sizes were calculated using Cohen's *d*, and interpreted using thresholds of <0.25, >0.25, >0.5, and >1.0 for trivial, small, moderate, and large, respectively (Rhea, 2004).

Results

Pre-exercise hydration status

Hydration status, measured as urine osmolality prior to each trial, did not differ between trials [KE, 420 (259, 581) mOsm·kg^{-1} vs. PLA, 460 (189, 732) mOsm·kg^{-1}; *P*=0.645, *d*=0.18].

Plasma βHB, glucose and lactate concentrations

Fasting plasma concentrations of βHB [KE, 0.11 (0.09, 0.13) mM; PLA, 0.11 (0.09, 0.13) mM; *P*>0.99], glucose [KE, 4.81 (4.62, 5.00) mM; PLA, 4.84 (4.57, 5.01) mM; *P*>0.99] and lactate [KE, 0.86 (0.71, 1.02) mM; PLA 0.96 (0.82, 1.11) mM; *P*>0.99] concentrations did not differ between trials (Figure 4.2). A main effect of time and condition (both *P*<0.001), and time x condition interaction effect (*P*<0.001) were observed for plasma βHB concentrations (Figure 4.2A). Ingestion of KE resulted in a rise in plasma βHB concentrations to 1.05 (0.83, 1.26) mM *(P*<0.001) by the start of exercise. Concentrations

continued to rise throughout exercise with the highest concentrations during KE observed at cessation of shuttle run to exhaustion at 2.61 (2.03, 3.10) mM *(P*<0.001).

A main effect of time (*P*<0.001) and condition (*P*=0.020) were observed for plasma glucose concentrations (Figure 4.2B). Plasma glucose concentrations were lower during KE compared to PLA at each time point with the exception of block 3, but *post-hoc* pairwise comparisons did not reveal significant differences between conditions at any time point. However, standardized differences in the mean indicated moderate effect sizes at each of these time points. Specifically, plasma glucose concentrations were lower during KE compared to PLA by 10.9% [-0.56 (-1.48, 0.35) mM; *d*=0.52] after block 1, 7.5% [-0.48 (-1.40, 0.44) mM; *d*=0.56] after block 2, 11.6% [-0.76 (-1.68, 0.15) mM; *d*=0.80] after block 4, and 8.4% [-0.55 (-1.46, 0.37) mM; *d*=0.56] after block 5. There was no difference in plasma glucose concentration at the end of the shuttle run to exhaustion [KE, 6.49 (6.01, 6.90) mM; PLA, 6.46 (5.64, 7.23) mM; *P*>0.99; *d*=0.04].

A main effect of time and condition (both *P*<0.001), and time x condition interaction effect (*P*=0.009) were observed for plasma lactate concentrations (Figure 4.2C). Plasma lactate concentrations were elevated from block 1 onwards during both conditions but were lower during KE compared to PLA at each time point. *Post-hoc* pairwise comparisons revealed significant differences between conditions at block 4 (*P*=0.037), block 5 (*P*=0.042) and the end of the shuttle run to exhaustion (*P*<0.001), and effect sizes indicating small, moderate and large effects across all time points i.e. block 1: -11.9%, -0.48 mM (-1.46, 0.50), *d*=0.27; block 2: -13.8%, -0.56 (-1.54, 0.42) mM, *d*=0.33; block 3: -20.2%, -0.73 (-1.71, 0.25) mM, *d*=0.45; block 4: -29.3%; -1.02 (-2.00, -0.04) mM, *d*=0.58; block 5: -30.1%, -1.00 (-1.98, 0.02) mM, *d*=0.63; end of the shuttle run to exhaustion: -21.5%, -1.85 (-2.83, -0.87) mM, *d*=1.00).

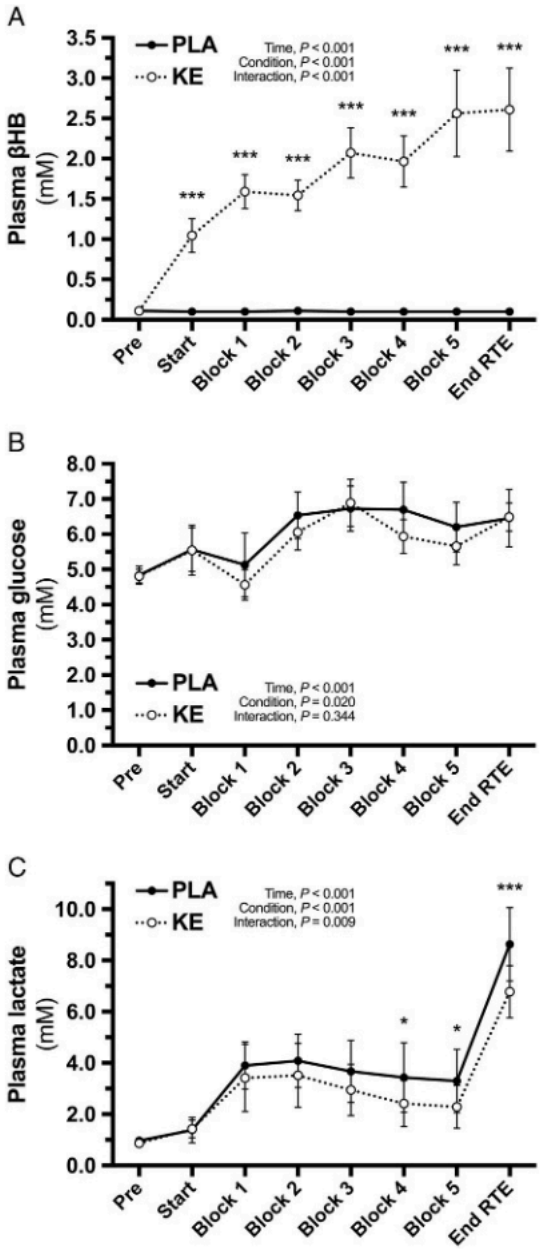

Figure 4.2 Plasma βHB (A), glucose (B), and lactate (C) responses to ketone ester and placebo ingestion.

Data are presented as mean values, with error bars representing 95% confidence intervals. * P < 0.05 KE vs. PLA; ***P < 0.001 KE vs. PLA; KE, ketone ester; PLA; placebo

For both HR and RPE, main effects of time were observed (both P<0.001), but the absence of main effects of condition or time x condition interaction effects indicates that ingestion of KE did not alter the HR or RPE response during any block of Part A of the LIST protocol (Figure 4.3). However, the average HR during the shuttle run to exhaustion was

lower [-3.9 (-6.4, -1.4) bpm; *P*=0.007; *d*=0.42] during KE [170.7 (163.4, 177.9) bpm] compared to PLA [174.6 (168.3, 180.8) bpm].

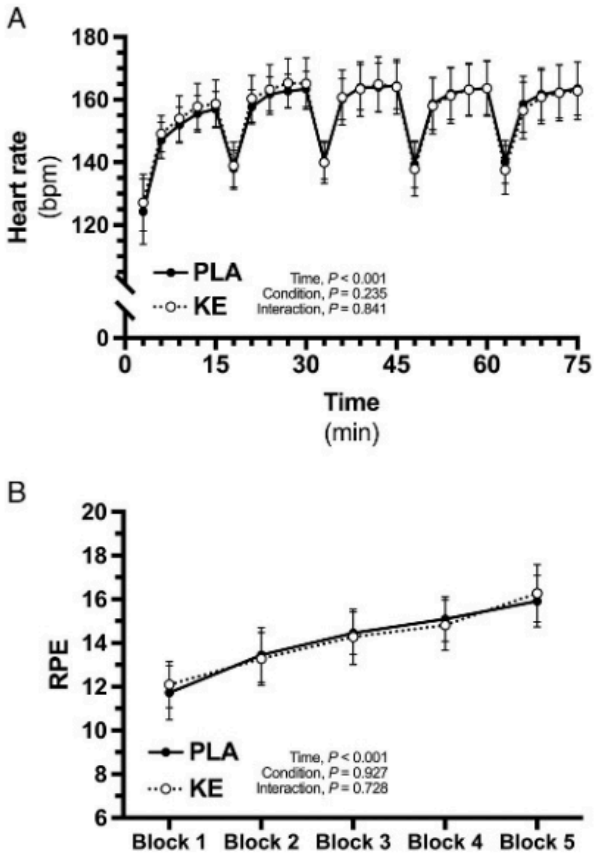

Figure 4.3 HR (A) and RPE (B) responses to ketone ester and placebo ingestion
Data are presented as mean values, with error bars representing 95% confidence intervals. KE, ketone ester; PLA; placebo.

15 m sprint times and shuttle run time to exhaustion

A main effect of time was observed for 15 m sprint times during Part A (*P*<0.001), but no main effect of condition or time x condition interaction effect were observed (Figure 4.4A). There was no statistically significant difference in the shuttle run time to exhaustion [KE, 229 (178, 280) sec; PLA, 267 (199, 336) sec; *P*=0.126] but standardized differences in the mean indicated a small effect size for this difference [-38 (-89, 13) sec; *d*=0.45].

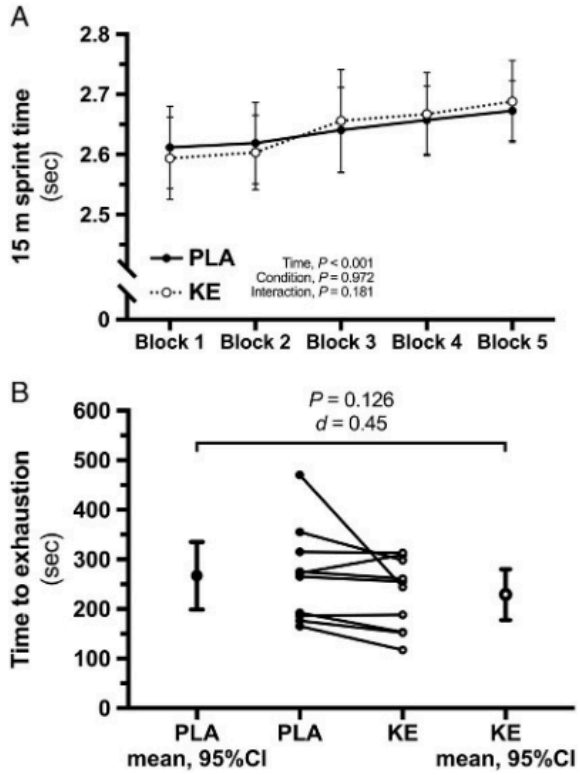

Figure 4.4 Fifteen-meter sprint times (A) and shuttle run time to exhaustion (B) during ketone ester and placebo trials

Data are presented as mean values, with error bars representing 95% CI. KE; ketone ester; PLA; placebo.

Cognitive performance

A time x condition interaction effect (P=0.021) was observed for the number of incorrect responses in the executive function multi-tasking test, which increased from pre- to post-exercise in PLA [1.8 (-0.6, 4.1)], but not in KE [0.0 (-1.8, 1.8)] (P=0.017; d=0.70) (Table 4.1). The absence of main effects for time or condition, and time x condition interaction effects indicates that there was no difference between conditions in either reaction time, or rapid visual information processing assessed by a sustained attention task (Table 4.1).

Gastrointestinal symptoms

Four out of eleven (36%) participants reported symptoms of GI distress during PLA and comprised of four (36%), three (27%), three (27%), one (9%) and one (9%) reports of belching, cramps, flatulence, boating and nausea, respectively. Nine out of eleven (82%) participants reported symptoms of GI distress during KE. These comprised of seven (64%), six (55%), four (36%), three (27%), three (27%) and one (9%) of the participants reporting nausea, cramps, belching, heartburn, flatulence and vomiting, respectively.

Identification of KE and best performance trials

Eight out of eleven (73%) participants correctly identified the trial in which they received KE, identifying KE by the awareness of taste and GI symptoms. However, only five (45%) of the participants correctly identified the trial in which they performed better in the Part B run to exhaustion. Only three participants (27%) stated that they believed KE ingestion improved their performance, and two out of those three participants correctly identified their KE trial and their best performance.

Table 4.1 Cognitive performance measures assessed before and after each trial

	Reaction time test (RTI)								
	Release time (msec)			Reaction time (msec)			Errors		
	Pre	**Post**	**Post-Pre**	**Pre**	**Post**	**Post-Pre**	**Pre**	**Post**	**Post-Pre**
KE	393 (360, 425)	394 (369, 419)	1 (16, 18)	222 (181, 263)	218 (177, 259)	-4 (-39, 31)	0.4 (-0.1, 0.8)	0.8 (0.0, 1.5)	0.4 (-0.6, 1.4)
PLA	404 (371, 436)	397 (368, 427)	-6 (-31, 19)	225 (172, 278)	237 (205, 269)	12 (-10, 34)	0.8 (-0.2, 1.7_	0.6 (-0.6, 1.8)	-0.1 (-1.0, 0.7)
d			-0.28			0.45			-0.46

	Multi-tasking test (MTT)								
	Response latency[##] (msec)			Correct responses[§]			Incorrect responses[§]		
	Pre	**Post**	**Post-Pre**	**Pre**	**Post**	**Post-Pre***	**Pre**	**Post**	**Post-Pre***
KE	590 (510, 669)	550 (483, 616)	-40 (-78, -2)	157.9 (156.4, 159.3)	157.9 (156.0, 159.7)	0.0 (-1.8, 1.8)	2.1 (0.7, 3.6)	2.1 (0.3, 4.0)	0.0 (-1.8, 1.8)
PLA	589 (526, 652)	543 (499, 587)	-46 (-74, -19)	157.8 (155.7, 159.8)	156.0 (152.8, 159.2)[†]	-1.8 (-4.1, 0.6)	2.3 (0.2, 4.3)	4.0 (0.8, 7.2)[†]	1.8 (-0.6, 4.1)
d			-0.16			-0.70			0.70

	Rapid visual information processing test (RVP)								
	Response latency (ms)			Correct responses			False alarms		
	Pre	**Post**	**Post-Pre**	**Pre**	**Post**	**Post-Pre**	**Pre**	**Post**	**Post-Pre**
KE	449 (389, 509)	430 (365, 495)	-19 (-65, 26)	44.0 (35.7, 52.3)	44.6 (36.9, 52.3)	0.6 (-2.7, 4.0)	2.0 (0.9, 3.1)	2.3 (0.5, 4.0)	0.3 (-0.9, 1.4)
PLA	460 (395, 425)	446 (383, 510)	-14 (-43, 16)	45.3 (39.0, 51.5)	44.8 (37.4, 52.1)	-0.5 (-5.7, 4.7)	2.4 (1.1, 3.6)	2.5 (0.8, 4.2)	0. (-1.2, 1.4)
d			0.12			-0.21			-0.08

Data are presented as mean (95%CI), n=8. Effect size calculated as Cohen's d. Symbols are $^{\#\#}P < 0.01$ for main effect of Time; $^{\S}P < 0.05$ for Condition x Time interaction effect; $^{\dagger}P < 0.05$ for Post vs. Pre; $^{*}P < 0.05$ for KE vs. PLA.

Discussion

The aim of the present study was to investigate the effect, if any, of the acute ingestion of a ketone ester on metabolic responses, physical and cognitive performance in team sport athletes in response to an intermittent running protocol that simulated soccer play. Compared to carbohydrate ingestion alone (PLA), ingestion of the ketone ester with carbohydrate (KE) resulted in an elevation in plasma βHB concentration to >1.5 mM after 15 min of exercise and reached ~2.6 mM by the end of exercise. Metabolic consequences included reductions in plasma glucose and lactate concentrations compared to PLA, but no differences in HR or RPE were observed between conditions. KE was without benefit to 15 m sprint times throughout the simulated protocol, or endurance capacity measured by shuttle run to exhaustion. However, cognitive performance in a multi-tasking executive function test was preserved with KE, but declined during PLA.

Recent reports have investigated the effect of acute ingestion of exogenous ketone supplements on physical performance in endurance athletes (Cox et al., 2016; Rodger et al., 2017; Leckey et al., 2017). Ingestion of a βHB ketone monoester (KME) increased distance covered in a 30 min cycling time-trial by ~2% (411±162 m) (Cox et al., 2016), whereas in contrast, ingestion of an acetoacetate ketone diester (KDE) impaired performance in a 31.2 km cycling time trial by 2±1% (58.2 sec) (Leckey et al., 2017). The latter effect was explained by a reduction in average power output by 3.7%, and coincided with a high prevalence of GI distress. Additionally, ingestion of a βHB salt formulation had no effect on average power output during a 4 min maximal performance cycling test (Rodger et al., 2017). A key distinction between these studies is the form of exogenous ketone supplement ingested. Acute ingestion of KME produces plasma βHB concentrations of ~3.0 mM after 20 min, but the KDE and racemic ketone salts result in blood βHB concentrations only in the 0.3 to 0.6 mM range (Rodger et al., 2017; Leckey et al., 2017; Evans et al., 2017). While this

concentration range constitutes acute nutritional ketosis and is sufficient to impact the metabolic response to exercise (O'Malley et al., 2017; Evans et al., 2018), performance is unlikely to be impacted unless circulating βHB concentrations exceed 1.0 mM (Evans, Cogan and Egan, 2017). In the present study, the KE condition resulted in an elevation of plasma βHB concentrations to >1.5 mM after 15 min of exercise and reached ~2.6 mM by the end of exercise, which is broadly similar to previous work (Cox et al., 2016; Stubbs et al., 2017). Our participants ingested 750 $mg.kg^{-1}$ body mass split across three boluses with 50% ingested 20 min prior to commencing exercise, and the remainder split into aliquots of 25% ingested at 30 and 60 min of exercise, respectively. This feeding strategy mimics the previous work with KME ingestion and exercise performance, which resulted in plasma βHB concentrations of ~2 mM 20 min after ingestion, and ranged from ~2.0 to 3.0 mM throughout 90 min of exercise (Cox et al., 2016). In contrast, our participants only achieved these concentrations 65 min after ingestion of the initial bolus of KE, but these participants ingested KE in the postprandial state as opposed to the fasted state in the KME work. Ingestion of KME in the post-prandial state can attenuate the C_{max} of blood βHB concentrations by 33%, and the 4 h βHB AUC by 27% (Stubbs et al., 2017).

Accordingly, despite the similar changes in circulating βHB concentrations to the previous investigation of performance effects using the KME (Cox et al., 2016), the KE condition in the present study was without benefit to 15 m sprint times throughout the 75 min intermittent running protocol (LIST Part A), or on shuttle run time to exhaustion (LIST Part B). The metabolic consequences of KME ingestion were recapitulated herein, namely lower plasma glucose and lactate concentrations during KE compared to PLA. A 10% reduction in plasma glucose was observed 35 min after ingestion of the initial KE bolus, and was 8 to 12% lower (moderate effects) during blocks 1, 2, 4 and 5 of Part A. The glucose-lowering effect of exogenous ketones is well-documented whether ingested alone (Cox et al., 2016; Stubbs et al.,

2017; O'Malley et al., 2017; Evans et al., 2018; Stubbs et al., 2018) or in combination with carbohydrate and/or protein (Stubbs et al., 2017; Cox et al., 2016; Vandoorne et al., 2017; Leckey et al., 2017; Myette-Côté et al., 2018). While the insulinotropic action of ketone bodies is not always observed (Nair et al., 1988; Mikkelsen et al., 2015), it can occur under certain conditions (Balasse & Fery, 1989), including when KME is ingested in the fasted state (Cox et al., 2016; Stubbs et al., 2017; Stubbs et al., 2018). However, when co-ingested with carbohydrate and/or protein, the glucose-lowering effect of exogenous ketones occurs despite similar circulating insulin concentrations in response to carbohydrate and/or protein alone at rest (Myette-Côté et al., 2018), during exercise (Cox et al., 2016), and during recovery from exercise (Vandoorne et al., 2017). A βHB-mediated glucose-lowering effect is likely a result of an attenuation of hepatic gluconeogenesis and increase in hepatic glucose uptake (Mikkelsen et al., 2015). The rise in plasma lactate concentrations was attenuated during KE compared to carbohydrate ingestion alone, consistent with previous KME work (Cox et al., 2016). An attenuation in the exercise-induced rise in plasma lactate was previously explained by a reduction in glycolytic flux, sparing of muscle glycogen during exercise and an increased contribution of ketone bodies and intramuscular triglycerides to energy provision (Cox et al., 2016). Whereas a 50% reduction in the rise in plasma lactate was observed during a 60 min pre-load at 75% W_{max} and 30 min time-trial in trained cyclists (Cox et al., 2016), we observed a reduction ranging from ~10 to 30%. Given the lower aerobic fitness in our team sport athletes, the trained cyclists may have had a greater capacity to extract ketones from circulation and oxidise them as a substrate, resulting in a larger contribution towards total energy production and a greater reduction in glycolytic flux. This is because ketone bodies are transported across the skeletal muscle membrane by monocarboxylate transporters (MCT), which are most highly expressed in type I muscle fibres, and are increased in response to endurance exercise training (Thomas et al., 2012).

For that reason, we previously hypothesised that performance benefits of exogenous ketones are most likely to be realised in those individuals with high levels of aerobic fitness and higher proportions of type I muscle fibres and/or MCT expression (Evans, Cogan and Egan, 2017). A lower level of aerobic fitness and training status, and therefore ability to oxidise circulating ketones may be one explanation for the lack of performance benefit in the present work. That notwithstanding, our performance test was shorter (~2 to 6 min) and intermittent in nature, which may be another factor contributing to the contrasting results. Another explanation may relate to the proposed benefits of exogenous ketone supplements being via their glycogen sparing effect. Given that we employed an optimal carbohydrate-based fuelling on the day prior to and the day of each trial, our athletes may not have experienced glycogen depletion to an extent that the purported glycogen sparing would have benefited performance in Part B. In fact, standardized differences in the mean used to assess magnitudes of effects between KE and PLA indicate a small effect size for a decrement in performance with KE, so it would be remiss not to consider that the effect of exogenous ketones in this instance may have been to impair carbohydrate utilisation.

Nutrition strategies such as high fat feeding, ketogenic diets and exogenous ketone ingestion alter substrate utilisation during exercise, which generally results in lower rates of carbohydrate utilisation at moderate-to-high exercise intensities (Spencer et al., 2005; Burke, 2015, Pinckaers et al., 2017). Whether this shift in substrate utilisation reflects a sparing of muscle glycogen, which can then be utilised later in an exercise challenge, or instead reflects an impairment of muscle glycogen utilisation during such exercise intensities is a salient issue for alternative fuelling strategies. The mechanistic basis for reduced carbohydrate utilisation in the presence of exogenous ketones is proposed as an attenuation of glycolytic flux via inhibition of pyruvate dehydrogenase (PDH) and phosphofructokinase by increases in NADH:NAD^+, acetyl-CoA:CoA or citrate. A similar mechanism is likely to contribute to

the impaired performance during moderate-to-high intensity efforts observed under high fat feeding (Havemann et al., 2006; Burke et al., 2017). The attenuation of PDH activity under such conditions (Stellingwerff et al., 2006) could be problematic for intermittent activity sports that require high intensity efforts, which rely heavily on ATP provision from glycolytic pathways, performed on a moderate intensity background. Clearly this is the nature of the exercise challenge in the present study, but future work will require direct measurement of PDH activity and glycolytic flux in muscle biopsies in order to make definitive conclusions about the effects of exogenous ketones on utilisation of muscle glycogen in this model. Conversely, we observed no benefit or decrement on 15 m sprint times performed at a rate of approximately nine sprints per 15 min block across 75 min of intermittent activity. Maximal short duration sprints rely primarily on the ATP-phosphocreatine (PCr) system and anaerobic glycolysis for energy provision, but as the number of repeated sprints increases, the contribution of both decline and the contribution of aerobic glycolysis of circulating glucose and muscle glycogen increases over time (Gaitanos et al., 1993; Parolin et al., 1999). The lower plasma lactate concentrations in Part A during KE suggests a reduction in glycolytic flux, but the reduction ultimately did not impact performance in repeated sprints of <3 sec duration.

A higher incidence of gastrointestinal (GI) symptoms occurred during KE compared to PLA, although this did not affect the HR or RPE responses during exercise. GI symptoms are a common side effect of KME and KDE ingestion and more work is needed on the dose and timing of both these supplements to mitigate this response. KME ingested as part of meal replacement milkshake drink causes a step-wise increase in symptoms with increasing dosages (Clarke et al., 2012). Furthermore, ingestion of 500 $mg.kg^{-1}$ body mass of KDE split in two doses caused symptoms in all participants during a cycling time-trial (Leckey et al., 2017). These symptoms are likely to be a large contributor to the performance decrement in

that study given the participants' nomination of their symptoms as a distraction or interference to performance. The incidence of GI symptoms was higher in the present study than in previous work with KME (Cox et al., 2016), but the aforementioned commencement of exercise in a fed as opposed to fasted state, or this protocol involving running as opposed to cycling exercise, may be contributing factors.

A novel finding herein is the preservation of executive function during KE compared to PLA, measured by the number of incorrect responses to a multi-tasking test. Given that team sport athletes are presented with a multitude of decisions throughout match play, interventions that preserve or improve cognitive performance could positively influence performance outcomes. The primary physiological role of ketogenesis as a survival mechanism during low carbohydrate availability is providing a substrate to the brain in the presence of diminishing blood glucose concentrations (Owen et al., 1967). Cognitive benefits and a neuroprotective role are established for exogenous ketones in non-exercise contexts (Ari et al., 2016; Murray et al., 2016; Kovacs et al., 2017; Svart et al., 2018). Notably, in a short-term (5 day) feeding study, rats supplemented daily with KME were 38% faster at completing a radial maze task, and made more correct decisions before making a mistake during the test (Murray et al., 2016). This outcome is consistent with our findings and suggests that central effects may be relevant during exercise, although other tests of cognitive function, i.e. reaction time and sustained attention tasks, were unaffected.

In conclusion, in team sport athletes acute ingestion of a ketone ester elevated plasma βHB concentrations, but did not improve performance in a shuttle run to exhaustion performed after 75 min of intermittent running. Reductions in plasma glucose and attenuated increases plasma lactate during exercise demonstrate the obvious effects of exogenous ketone ingestion on carbohydrate metabolism during exercise. However, participants experienced incidences of GI symptoms. These results underscore the need for future work to explore

possible dose-response effects while minimising any GI distress to athletes. Despite the lack of benefit to physical performance, the novel finding of preserved executive function after exhausting exercise suggests that there remains a possibility that exogenous ketones could enhance sport-specific performance of team sport athletes via other mechanisms.

Chapter 5

Presented in manuscript format as submitted for review to Medicine and Science in Sports and Exercise in February 2019 (Appendix D).

Evans M, McSwiney F, Brady A, Egan B. No benefit of ingestion of a ketone monoester supplement on 10-km running performance (currently under review)

Abstract

Introduction: Pre-exercise ingestion of exogenous ketones alters the metabolic response to exercise, but effects on exercise performance have been equivocal. **Methods:** On two occasions in a double-blind, randomized crossover design, eight endurance-trained runners performed 1 h of submaximal exercise at ~65% $\dot{V}O_{2peak}$ immediately followed by a 10-km self-paced TT on a motorized treadmill. An 8% carbohydrate-electrolyte solution was consumed before and during exercise, either alone (CHO+PLA), or with 573 mL kg^{-1} of a ketone monoester supplement (CHO+KME). Expired air, heart rate (HR), and rating of perceived exertion (RPE) were monitored during submaximal exercise. Serial venous blood samples were assayed for plasma glucose, lactate and β-hydroxybutyrate concentrations. **Results:** CHO+KME produced plasma β-hydroxybutyrate concentrations of ~1.0 to 1.3 mM during exercise ($P < 0.001$), but plasma glucose and lactate concentrations were similar during exercise in both trials. $\dot{V}O_2$, running economy, respiratory exchange ratio, HR and RPE did not differ between trials. Performance in the 10-km TT was not different ($P = 0.483$) between CHO+KME (mean = 2402 s; 95% confidence interval [CI] = 2204, 2600 s) and CHO+PLA (mean = 2422 s; 95% CI = 2217, 2628 s). Cognitive performance, measured by reaction time and a multi-tasking test, did not differ between trials. **Conclusion:** Compared with carbohydrate alone, co-ingestion of KME by endurance-trained athletes elevated plasma β-hydroxybutyrate concentrations, but did not improve 10-km running TT or cognitive performance.

Introduction

The therapeutic and performance potential of exogenous ketone supplements has been the subject of increasing interest in recent years (Egan & D'Agostino, 2016; Koutnik et al., 2018). Metabolic effects the ketone bodies (KB), namely β-hydroxybutyrate (βHB), acetoacetate (AcAc), are well-established in many organs, including attenuation of glycolysis, hepatic glucose output and adipose tissue lipolysis (Robinson & Williamson 2018), but their potential role in modulating substrate utilization has garnered attention for athletic performance (Cox et al., 2016; Evans & Egan, 2018). In the fasted state, KB provide up to 10% of energy to skeletal muscle during exercise (Balasse & Fery, 1989), and after acute ingestion of exogenous ketone supplements, this contribution can increase to 16 to 18% when circulating βHB is elevated to the 3 to 4 mM range (Cox et al., 2016). Moreover, this increase in βHB oxidation coincides with a reduction in glycolytic flux, as evidenced by an attenuation in the exercise-induced rise in plasma lactate and glycolytic intermediates, and an increase in intramuscular triglyceride utilization during exercise (Cox et al., 2016).

Circulating KB concentrations are <0.1 mM in the postprandial state, whereas hyperketonaemia is accepted as KB concentrations exceeding 0.2 mM (Robinson & Williamson 1980). Ingestion of a variety of exogenous ketone supplements can acutely produce nutritional ketosis (Cox et al., 2016; Evans et al., 2018; Stubbs et al., 2018; Stubbs et al., 2017; Rodger et al., 2017; O'Malley et al., 2017; Leckey et al., 2017; Myette-Côté et al., 2018; Waldman et al., 2017; Vandoorne et al., 2017; Holdsworth et al., 2017; Fischer et al., 2018; Evans & Egan, 2018), which has been defined as circulating KB concentrations >0.5 mM (Volek et al., 2015). The most potent of these exogenous ketone supplements is the (R)-3-hydroxybutyl (R)-3-hydroxybutyrate ketone monoester (KME). When ingested at rest in the fasted state, KME produces a dose-dependent increase in circulating βHB concentrations of up to 6 mM 20 min after the ingestion of up to 573 $mg.kg^{-1}$ body mass (Cox et al., 2016;

Stubbs et al., 2018). This elevation in βHB concentration coincides with decreases in plasma glucose, free fatty acids, triglycerides and ghrelin concentrations (Cox et al., 2016; Stubbs et al., 2017; Stubbs et al., 2018 Myette-Côté et al., 2018). Exercise attenuates the rise in βHB concentrations, as ingestion of 573 $mg.kg^{-1}$ body mass KME prior to 45 min cycling at 45% and 75% peak power output (W_{max}) resulted in circulating βHB of ~4.0 mM and ~3.0 mM, respectively. As a consequence of the aforementioned effects on substrate utilization, acute ingestion of KME attenuates the rise in plasma glucose and lactate concentrations during exercise, whether in an endurance cycling or intermittent running context (Cox et al., 2016; Evans & Egan, 2018).

These metabolic consequences have been proposed to explain the observation that the co-ingestion of KME in addition to a carbohydrate-based fuelling strategy improved performance in a 30 min maximum distance cycling time-trial by 2% when preceded by 1 h of submaximal 'pre-load' exercise (Cox et al., 2016). In contrast, high-intensity shuttle running capacity (~4 to 6 min) performed after 75 min of intermittent running was not improved in team sport athletes with KME co-ingestion compared to carbohydrate alone (Evans and Egan, 2018). While the former study considering a 'sparing' of muscle glycogen to be major factor in the performance benefit (Cox et al., 2016), the latter study speculated that the attenuation of glycolytic flux in the presence of elevated circulating βHB may have been a factor in the lack of performance benefit in that exercise model (Evans & Egan, 2018). Performance in exercise of long duration that incorporates high intensity efforts (i.e. sprint finishes, climbs) is largely dependent on carbohydrate utilization (Hawley & Leckey, 2015). Therefore, nutrition strategies that could spare muscle glycogen and maintain high intensities in the latter parts of races are of interest to scientists and practitioners (Pinckaers et al., 2017). However, if glycogen sparing occurs via an attenuation of glycolytic flux that cannot be overcome when higher intensity efforts are required, this would instead be likely to impair

performance (Hawley & Leckey, 2017). Moreover, the recent observation that acute ingestion of KME prior to intermittent exercise in team sport athletes resulted in preserved executive function as measured by a decision-making task after volitional exhaustion (Evans & Egan, 2018), remains to be confirmed in other exercise settings.

Therefore, the aim of the present study was to investigate the effects of acute ingestion of an exogenous ketone supplement in the form of a commercially-available KME on physiological responses, physical and cognitive performance in endurance-trained runners in response to 1 h submaximal exercise immediately followed by a 10-km time trial. We hypothesise ingestion of KME will have no effect on physical or cognitive performance in endurance-trained runners.

Methods

Participants

Eight trained, middle and long distance runners (M/F, 7/1; age, 33.5±7.3 y; height, 1.79±0.07 m; body mass, 68.8±9.7 kg; body fat, 8.0 ± 4.1 %; $\dot{V}O_{2peak}$, 62.0±5.6 mL kg^{-1} min^{-1}) gave written informed consent to participate after written and verbal explanations of the procedures. Ethical approval (permit number: DCUREC2018_039) was obtained from the Dublin City University Research Ethics Committee in accordance with the Declaration of Helsinki.

Experimental design

Participants visited the laboratory for exercise tests on four separate occasions over a 21 to 28 day period, comprising one baseline, one familiarization and two main experimental trials. During their first visit to the lab, each participant's maximal rate of oxygen consumption ($\dot{V}O_{2peak}$) was determined using an incremental treadmill test to volitional exhaustion. The exercise protocol performed in the familarization visit (visit 2) and two main

experimental trials (visits 3 and 4) comprised of a pre-load of 1 h of treadmill running at 65%$\dot{V}O_{2peak}$ followed by a self-paced 10-km time-trial (TT) performance test performed on a motorized treadmill (Figure 5.1). A battery of cognitive tests were performed before and after the exercise protocol. The main experimental trials were performed in a double-blind, placebo-controlled, randomized crossover design. Visits 2, 3 and 4 were identical in terms of the pre-test preparation (standardized physical activity and diet for 24 h prior to each visit) and the exercise protocol. The visits differed only in the drinks consumed before and during exercise, namely an 8% carbohydrate-electrolyte solution, which was co-ingested with either a flavored placebo condition (CHO+PLA), or included the (R)-3-hydroxybutyl (R)-3-hydroxybutyrate ketone monoester (CHO+KME). The primary outcome was endurance performance measured by time to complete the self-paced 10-km TT, with secondary outcomes including cognitive performance, oxygen consumption ($\dot{V}O2$), running economy, respiratory exchange ratio (RER), heart rate (HR), rating of perceived exertion (RPE), and plasma βHB, glucose, and lactate concentrations.

Assessment of $\dot{V}O_{2peak}$ and submaximal running speeds

Body mass was measured to the nearest 0.2 kg using a calibrated digital scales (SECA, Hamburg, Germany), and height was measured to the nearest 0.01 m using a wall-mounted stadiometer (Holtain, Crymych, UK). Body fat was determined by bioelectrical impedance analysis (DC-430U Dual Frequency Analyzer; Tanita, Arlington Heights, IL USA). All exercise testing and experimental trials were conducted on a motorized treadmill (T200; COSMED, Rome Italy). Initially, for the determination of the responses in $\dot{V}O_2$ and blood lactate concentration at submaximal running speeds, participants ran for 4 min stages at a progressively increasing speeds, interspersed with a 1 min rest interval for determination of blood lactate concentrations (Lactate Pro 2; Arkray, Kyoto, Japan , RPE (Borg scale) and HR (Polar H7; Polar, Kempele Finland). The first stage was 4 km h^{-1} slower than the average

speed corresponding to each participant's personal best time for a 10-km race. For each subsequent stage, the running speed was increased by 1 km h^{-1} until the running speed exceeded the speed corresponding to their personal best 10-km race speed. After a 10 min rest, participants began running at a speed corresponding to the last completed speed of the preceding test. Treadmill speed was increased by 2.0 km h^{-1} every 2 min for two stages, after which treadmill gradient was increased by 1.0% every 1 min until volitional fatigue. Expired air was collected and analyzed throughout these tests using the Quark RMR metabolic cart (COSMED, Rome, Italy). $\dot{V}O_2$, carbon dioxide production ($\dot{V}CO_2$), and RER were calculated from an average of breath-by-breath measurements during the last 30 s of each stage during the submaximal running stages and the assessment of $\dot{V}O_{2peak}$. $\dot{V}O_{2peak}$ was considered to have been achieved if two of the following criteria were achieved: (1) plateauing of $\dot{V}O_2$ despite increasing treadmill speed (increase of less than 2.0 mL kg^{-1} min^{-1}), (2) HR within 5% of the age-predicted HR_{max} (208 – 0.7 x age in years), and (3) an RER ≥1.10.

Cognitive test battery

The battery of cognitive tests (CANTAB Cognition, Cambridge, UK) was administered via a touch screen tablet lasting ~10 min. An identical test battery was administered before and after each trial in visits 2, 3 and 4.

During the reaction time (RTI) test, participants select and hold a button at the bottom of the screen and five circles are presented above. In each case, a yellow dot appears in one of the five circles, and the participants must react as soon as possible, releasing the button at the bottom of the screen, and selecting the circle in which the dot appeared. Release time (msec), reaction time (msec), and number of errors were recorded.

The multi-tasking test (MTT) is a test of executive function that measures the participant's ability to switch attention between stimuli, and ignore task-irrelevant information. White arrows are displayed on a black background, with the arrows located on

either the left or right side of the screen, and pointing either to the left or to the right. A cue is displayed at the same time as the arrows, reading either "SIDE" or "DIRECTION". When the "SIDE" cue is presented, the participant is required to press a button on the left or right of the screen corresponding to the side of the screen where the arrow is presented, regardless of the direction the arrow is pointing. Conversely, when the "DIRECTION" cue is presented, the participants are required to touch a button on the left or right of the screen corresponding to the direction the arrow is pointing, regardless of which side of the screen the arrow is presented. Reaction time (msec), and number of correct and incorrect responses were recorded.

Pre-trial preparation

All experimental trials commenced between 0730 and 1130, and were completed within a period of 4.0-4.5 h (Figure 5.1). On an individual basis, participants performed their second main experimental trial at the same time ±1 h as their first main trial. Pre-trial preparation was the same for the familiarization visit and each main experimental trial. Participants were asked to abstain from alcohol for 48 h and caffeine for 24 h, and refrain from strenuous exercise training on the day prior to each trial. For the day prior to experimental trials, participants were provided with a prescribed meal plan that provided ~2800 kcal (~41 kcal.kg^{-1}) at a macronutrient ratio of 60% carbohydrate (~6.2 g kg^{-1}), 20% protein and 20% fat. Participants performed the two main experimental trials separated by either 7 or 14 days.

Main experimental trials

The protocol for the familiarization and main experimental trials were identical except for the drinks consumed before and during exercise (Figure 5.1). Participants arrived to the laboratory in a fasted state 2 h prior to the commencement of exercise, and immediately consumed a standardized breakfast of quick-cook porridge oats and cereal bars providing

~300-400 kcal (~4.4-5.8 kcal.kg^{-1}) and ~1.0 g kg^{-1} of carbohydrate, and 500 mL of water. Participants proceeded to complete the cognitive test battery 45 min after breakfast. Thereafter, an indwelling catheter (21G Insyte Autoguard; Becton Dickinson, Franklin Lakes, NJ USA) was introduced into an antecubital vein for serial blood sampling at rest (-30 and 0 min), during submaximal exercise (20, 40 and 60 min) and immediately after the 10-km TT.

For each trial, a bolus of a given drink was ingested 30 min prior to exercise (drink 1), at 20 min intervals during the 1 h of submaximal running (drinks 2 to 4), and at the 5-km mark of the 10-km TT (drink 5) (Figure 5.1). The carbohydrate-based fuelling strategy (CHO) consisted of a 6.4% carbohydrate-electrolyte solution (Lucozade Sport; Lucozade Ribena Suntory Ltd., Uxbridge, UK) with maltodextrin (Cargill Inc, Minneapolis, MN USA) added to make an 8.0% carbohydrate-electrolyte solution that was provided at a rate of ~1.0 g min^{-1} of exercise. During CHO+PLA, CHO was supplemented with denatonium benzoate, malic acid and arrow root extract to mimic the bitter taste and mouth-feel of the KME. During CHO+KME, CHO was supplemented with 573 mg.kg^{-1} body mass of a (R)-3-hydroxybutyl (R)-3-hydroxybutyrate ketone monoester (HVMN^{TM} Ketone; HVMN, Inc., San Francisco, CA USA). The commercially-available ketone ester was mixed directly with the carbohydrate-electrolyte solution for ingestion, and the 573 mg.kg^{-1} body mass dose was divided into three boluses at a ratio 50:25:25 ingested at -30 min (drink 1), 20 min (drink 2) and 60 min (drink 4), respectively (Figure 5.1). During CHO+PLA, drinks 1, 2 and 4 were flavored with the bitter additives to taste match with CHO+KME, and in both trials, drinks 3 and 5 were provided as the unadulterated 8% carbohydrate-electrolyte solution. All drinks were administered in opaque drinks bottles.

For the exercise protocol, participants first performed a standardized 5 min warm up on the motorized treadmill (8 km h^{-1}) followed by self-selected stretching. Participants then performed 1 h of treadmill running at a speed corresponding to ~65%$\dot{V}O_{2peak}$ (Table 5.1).

Immediately after completion of the 1 h pre-load, participants completed a 10-km TT. The pre-load followed by TT protocol was modeled on the previous work demonstrating a benefit of KME on cycling TT performance (Cox et al., 2016), and has been similarly applied to treadmill running in previous studies (Russell et al., 2004; Scott et al., 2019). Prior to each TT, participants were told to complete the distance as fast as possible and they accelerated from a standing start by manually-adjusting a mounted control panel on the side of the treadmill. Participants were blinded to the speed of the treadmill and the time elapsed at all times, but were aware of the distance covered throughout the TT, including the 5-km mark when drink 5 was provided. After completing the 10-km TT, participants completed the same cognitive test battery as completed prior to exercise.

Venous blood samples were collected at 30 min prior to exercise, at 20 min intervals during submaximal exercise, and immediately after the 10-km TT. HR and RPE were recorded at 20 min intervals during submaximal exercise. Expired air was collected during the first 10 min, 25 to 30 min, and 55 to 60 min of the submaximal exercise for the monitoring of exercise intensity, and calculation of RER and running economy. Running economy is expressed as the volume of oxygen required to run 1 km relative to body mass (mL kg^{-1} km^{-1}) (Barnes & Kilding, 2015). Incidences of gastrointestinal (GI) symptoms were recorded by interview after each trial. At the end of visit 4, participants completed an exit interview in which they were asked whether they could identify the CHO+KME condition, and to identify which experimental trial they believed that they performed their best TT.

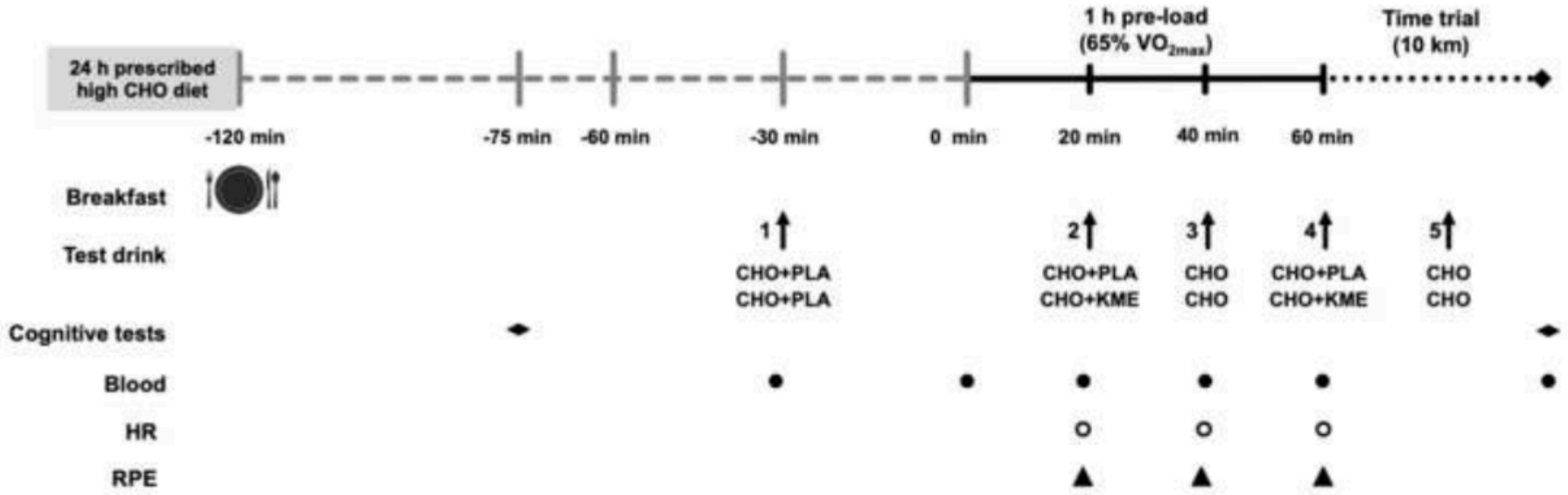

Figure 5.1 Schematic of the study protocol.

CHO, carbohydrate electrolyte solution; HR, heart rate; KME, ketone monoester; PLA, placebo; RPE, rating of perceived exertion

Blood analysis

Blood was collected in plastic tubes (2 mL) containing sodium heparin (Plus Blood Collection Tubes; Becton Dickinson, Franklin Lakes, NJ USA) for subsequent analysis of βHB. A second blood sample was collected in plastic tubes (4 mL) containing sodium fluoride (Plus Blood Collection Tubes; Becton Dickinson, Franklin Lakes, NJ USA) for subsequent analysis of glucose and lactate. All collection tubes were pre-chilled, and blood samples were stored on ice before centrifugation at 3000 *g* for 10 min at 4ºC, after which aliquots of plasma were separated for storage at -80ºC until later analysis. Plasma βHB was determined by colormetric assay as per the manufacturer's instructions (MAK041; Sigma-Aldrich, Arklow, Ireland). Plasma glucose and lactate were measured using the RX Daytona^{TM} chemical autoanalyser and appropriate reagents as per the manufacturer's instructions (Randox Laboratories, Crumlin, UK: assay codes GL3815 and LC3980, respectively).

Statistical analysis

Data were evaluated using Prism v8.0 (GraphPad Software, Inc., San Diego, CA USA) and are presented as mean [lower, upper 95% confidence interval (CI) of the mean], except for the participant characteristics, which are described as mean±SD. A paired samples t-test was used to determine differences between trials in time to complete the 10-km TT. The smallest worthwhile difference (SWD) was set at 0.2 between-subject SD, which is suggested to represent a practically-relevant change in performance in athletes (Hopkins et al., 2009). Thus, SWD corresponded to 48 sec, or 2.0%, for 10-km TT performance in this study. Two-way (time x condition) repeated measures of analysis variance (ANOVA) was used to determine differences between the two experimental trials for all variables with serial measurements. When a main effect of condition, or an interaction effect between condition and time was indicated, *post-hoc* testing was performed with Bonferroni's correction with

multiplicity-adjusted *P* values applied to compare CHO+KME to CHO+PLA at the respective time points. The data were tested for normality using Shapiro-Wilk test prior to proceeding with the parametric tests described. For null hypothesis statistical testing, the significance level was set at $\alpha = 0.05$ for all tests.

Results

Plasma βHB, glucose and lactate concentrations

Postprandial plasma concentrations of βHB (mean [95% CI]: CHO+KME, 0.27 [0.22-0.33] mM; CHO+PLA, 0.28 [0.14-0.43] mM), glucose (CHO+KME, 3.96 [3.22-4.70] mM; CHO+PLA, 3.70 [3.06-4.35] mM), and lactate (CHO+KME, 1.04 [0.79-1.29] mM; CHO+PLA, 1.02 [0.84-1.20] mM) did not differ between trials (all $P > 0.99$). A main effect of time and condition (both $P < 0.001$) and a time-condition interaction effect ($P < 0.001$) were observed for plasma βHB concentrations (Figure 5.2A). Ingestion of CHO+KME resulted in a rise in plasma βHB concentrations to 0.99 (0.85-1.14) mM at 0 min. βHB concentrations peaked at 1.33 (1.13-1.52) mM during submaximal exercise at 40 min, with similar concentrations observed at the cessation of the 10-km TT at 1.33 mM (0.95-1.70) mM.

A main effect of time ($P < 0.001$) and condition ($P = 0.027$) was observed for plasma glucose concentrations (Figure 5.2B). Plasma glucose concentrations were lower in CHO+KME at 0 min, i.e. 30 min after ingestion of the first bolus of either CHO+KME or CHO+PLA (CHO+KME, 3.87 [3.22-4.70] mM; CHO+PLA, 4.52 [3.91-5.13] mM; $P = 0.016$) (Figure 5.2B). Plasma glucose concentrations rose throughout submaximal exercise (Figure 5.2B) with the highest concentrations observed at cessation of the 10-km TT (CHO+KME, 6.94 [5.60-8.28] mM; CHO+PLA 7.24 [5.93-8.54] mM), with no difference between trials ($P > 0.99$).

A main effect of time ($P < 0.001$) was observed for plasma lactate concentrations, but were similar between trials at all time points (Figure 5.2C). Peak plasma lactate concentrations were observed at cessation of the 10-km TT (CHO+KME, 6.94 [4.15, 9.73] mM; CHO+PLA, 7.48 [5.46-9.51] mM; $P = 0.738$).

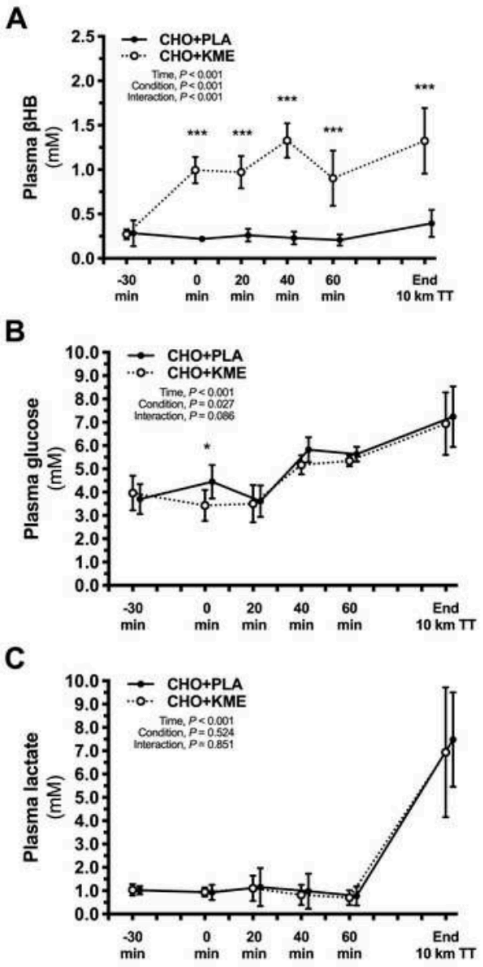

Figure 5.2 Plasma βHB (A), glucose (B), and lactate (C) concentrations during ketone monoester and placebo trials

Data are presented as mean values, with error bars representing 95% confidence intervals. *P < 0.05 for CHO+KME vs. CHO+PLA; ***P < 0.001 for CHO+KME vs. CHO+PLA. CHO+PLA, placebo; CHO+KME, ketone monoester

Submaximal exercise

Running speeds were identical between trials as per the study design. There was no difference in $\%\dot{V}O_{2peak}$, $\dot{V}O_2$, running economy, $\dot{V}CO_2$, RER, HR, and RPE between CHO+KME and CHO+PLA during the submaximal exercise period (Table 5.1). Main effects of time were observed for the decline in RER (P < 0.001), and the increase in RPE (P < 0.001) during the submaximal exercise bout (Table 5.1).

Table 5.1 Physiological responses to 1 h of treadmill running at ~65% $\dot{V}O_{2peak}$ with carbohydrate co-ingested with either placebo or a ketone monoester

	Time			
	0-10 min	**10-30 min**	**30-60 min**	***P* value**
Running speed (km h^{-1})	12.4 (11.3, 13.5)	12.4 (11.3, 13.5)	12.3 (11.1, 13.5)	
$\dot{V}O_2$ (L min^{-1})				Time, $P = 0.517$
CHO+PLA	2.84 (2.52, 3.16)	2.84 (2.56, 3.12)	2.81 (2.53, 3.09)	Condition, $P = 0.153$
CHO+KME	2.78 (2.42, 3.13)	2.79 (2.42, 3.13)	2.72 (2.49, 2.95)	Interaction, $P = 0.700$
%$\dot{V}O_{2peak}$				Time, $P = 0.576$
CHO+PLA	67.0 (62.8, 71.2)	66.9 (64.5, 69.4)	66.2 (63.8, 69.4)	Condition, $P = 0.170$
CHO+KME	65.3 (60.9, 69.8)	65.8 (62.6, 69.8)	64.1 (63.2, 65.0)	Interaction, $P = 0.710$
Running economy (mL kg^{-1} km^{-1})				Time, $P = 0.633$
CHO+PLA	202 (184, 219)	203 (185, 220)	202 (185, 219)	Condition, $P = 0.182$
CHO+KME	196 (181, 212)	199 (181, 217)	196 (179, 213)	Interaction, $P = 0.779$
$\dot{V}CO_2$ (L min^{-1})				Time, $P = 0.058$
CHO+PLA	2.67 (2.36, 2.99)	2.60 (2.30, 2.90)	2.55 (2.26, 2.84)	Condition, $P = 0.470$
CHO+KME	2.63 (2.28, 2.98)	2.58 (2.28, 2.89)	2.50 (2.26, 2.74)	Interaction, $P = 0.677$
RER				Time, $P < 0.001$***

CHO+PLA	0.94 (0.92, 0.96)	0.91 (0.89, 0.94)	0.91 (0.88, 0.93)	Condition, $P = 0.315$
CHO+KME	0.95 (0.92, 0.97)	0.92 (0.89, 0.96)	0.92 (0.89, 0.95)	Interaction, $P = 0.478$
HR (bpm)				Time, $P = 0.121$
CHO+PLA	141 (133, 149)	146 (137, 155)	145 (137, 154)	Condition, $P = 0.359$
CHO+KME	140 (131, 150)	144 (134, 154)	143 (134, 152)	Interaction, $P = 0.747$
RPE				Time, $P < 0.001$***
CHO+PLA	10 (9, 12)	11 (10, 13)	12 (10, 13)	Condition, $P = 0.903$
CHO+KME	10 (8, 12)	11 (9, 12)	11 (9, 13)	Interaction, $P = 0.656$

Data are presented as mean (95% CI), n = 8. ***$P < 0.001$.

10-km TT performance

There was no statistically significant difference in 10-km TT performance between trials (CHO+KME, 2402 [2204-2600] s; CHO+PLA, 2422 [2217-2628] s; $P = 0.483$) (Figure 5.3A). Compared to CHO+PLA, three participants demonstrated improvements in performance with CHO+KME that were greater than the SWD, and one participant demonstrated a decrement in performance with CHO+KME that was greater than the SWD (Figure 5.3B). The remaining participants' differences in performance between trials were less than the SWD. Running speeds for each 2 km split during the 10-km TT did not differ between trials, but did increase progressively throughout the TT (main effect of time, $P < 0.001$) (Figure 5.3C).

Cognitive performance

In the reaction time test (RTI), main effects of time ($P = 0.026$) and condition ($P = 0.026$) were observed for release time, but no interaction effect was present ($P = 0.535$), whereas an interaction effect was observed for reaction time ($P = 0.014$) (Table 5.2). In the multi-tasking test (MTT), a main effect of time was observed for response latency ($P = 0.010$), correct responses ($P = 0.049$) and incorrect responses ($P = 0.036$), but no main effects of time, or interaction effects were observed across these parameters (all $P > 0.05$) (Table 5.2). Overall, there was no difference in cognitive performance between conditions in either the RTI, or MTT assessments (Table 5.2).

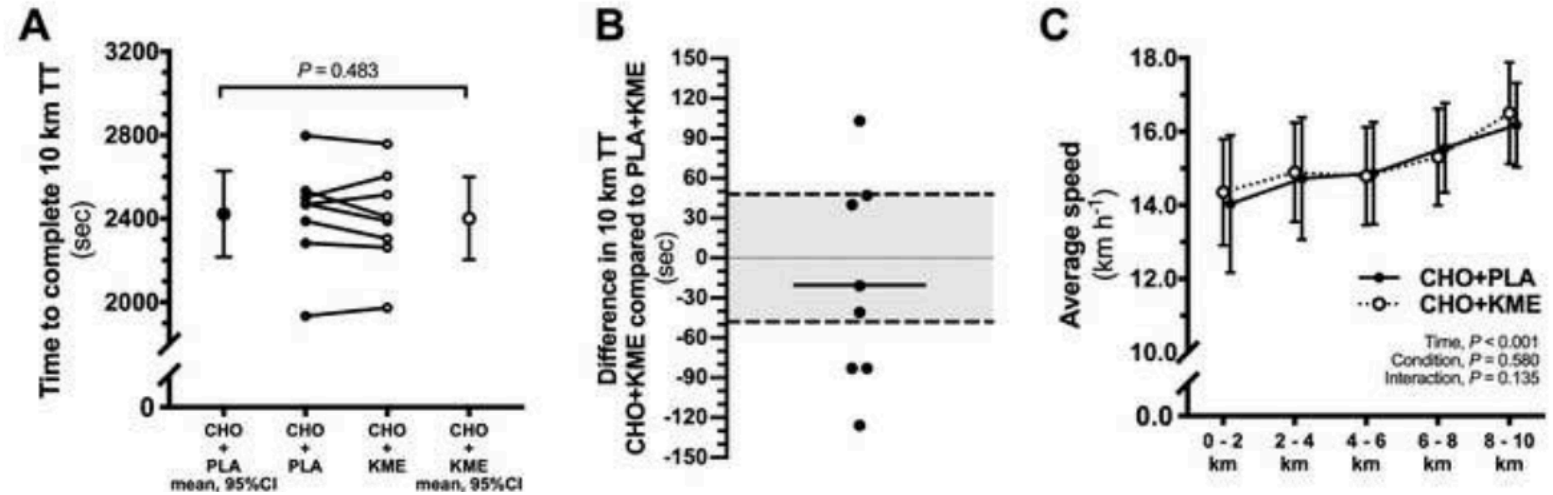

Figure 5.3 10-km time-trial performance (A), individual differences between CHO+KME compared to CHO+PLA (B), and running speeds for each 2 km split during the 10-km time-trials (C) in response to ketone monoester and placebo ingestion

Data in (A) and (C) are presented as mean values, with error bars representing 95% confidence intervals. The shaded area in (B) represents the range for the smallest worthwhile difference in 10-km time-trial performance in this cohort. CHO+PLA, placebo; CHO+KME, ketone monoester

Table 5.2 Cognitive performance in response to ketone monoester and placebo ingestion

	Reaction time test (RTI)								
	Release time[#,§] (msec)			Reaction time[†] (msec)			Errors		
	Pre	**Post**	**Post-Pre**	**Pre**	**Post**	**Post-Pre**	**Pre**	**Post**	**Post-Pre**
CHO+PLA	417 (373, 461)	401 (356, 446)	-16 (-35, 2)	223 (171, 276)	221 (165, 278)	-2 (-18, 14)	0.3 (-0.1, 0.6)	0.6 (0.0, 1.2)	0.4 (-0.4, 1.1)
CHO+KME	430 (383, 477)	409 (368, 450)[*]	-21 (-40, -2)	214 (176, 252)	232 (183, 282)[*]	18 (2, 34)	0.6 (-0.3, 1.5)	0.5 (0.0, 1.1)	-0.1 (-1.3, 1.1)

	Multi-tasking test (MTT)								
	Response latency[#] (msec)			Correct responses[#]			Incorrect responses[#]		
	Pre	**Post**	**Post-Pre**	**Pre**	**Post**	**Post-Pre**	**Pre**	**Post**	**Post-Pre**
CHO+PLA	599 (500, 698)	561 (447, 674)[***]	-38 (-58, -18)	159 (157, 160)	157 (155, 159)	-2 (-4, 1)	1 (0, 3)	3 (1, 5)	2 (-1, 4)
CHO+KME	583 (513, 653)	541 (461, 622)[***]	-41 (-62, -21)	158 (157, 160)	157 (156, 159)	-1 (-4, 2)	2 (0, 3)	3 (1, 4)	1 (-2, 4)

Data are presented as mean (95%CI), n=8. Symbols are $^{\#}P < 0.05$ for main effect of Time; $^{\S}P < 0.05$ for main effect of Condition; $^{\dagger}P < 0.05$ for Condition x Time interaction effect; $^{*}P < 0.05$ for Post vs. Pre; $^{***}P < 0.001$ for Post vs. Pre.

Gastrointestinal symptoms

Out of 8 participants, 4 (50%) reported symptoms of GI distress during CHO+PLA and comprised 4 (50%), 3 (38%), 1 (13%), 1 (13%), and 1 (13%) incidences of belching, flatulence, reflux, urge to defecate, and diarrhoea, respectively. Out of 8 participants, 5 (63%) reported symptoms of GI distress during CHO+KME and comprised 3 (38%), 2 (25%), 1 (13%), 1 (13%), 1 (13%), and 1 (13%) incidences of belching, urge to defecate, cramps, reflux, nausea, and stitch, respectively.

Identification of CHO+KME and best performance trials

Out of 8 participants, 2 (25%) correctly identified the trial in which they received CHO+KME, identifying CHO+KME by taste and a perceived alteration of performance. Six (75%) participants declared that they could not differentiate between CHO+PLA and CHO+KME. Seven (88%) participants correctly identified the trial in which they performed their best 10-km TT.

Discussion

The present study investigated whether the acute ingestion of a commercially-available ketone monoester supplement altered metabolic responses, physical and cognitive performance in endurance-trained runners in response to 1 h of submaximal exercise immediately followed by a treadmill-based self-paced 10-km TT. Compared with placebo (CHO+PLA), ingestion of the ketone monoester (CHO+KME) elevated plasma β-hydroxybutyrate to ~1.0 mM at the onset of submaximal exercise, and reached ~1.3 mM at the end of the 10-km TT. However, CHO+KME did not alter the metabolic or

cardiorespiratory responses to exercise, or demonstrate benefit to physical or cognitive performance compared to CHO+PLA ingestion.

The present study adds to the growing body of literature investigating the effects on exercise performance of elevating ketone body concentrations by exogenous means. The term "exogenous ketone supplement" encompasses a range of different forms of supplements, with each having differential effects on the metabolic response to exercise, and exercise performance. These studies have included the acute ingestion of a (R)-3-hydroxybutyl (R)-3-hydroxybutyrate ketone monoester (KME) (Cox et al., 2016; Evans & Egan, 2018), and R,S-1,3-butanediol acetoacetate ketone diester (KDE) (Leckey et al., 2017), racemic ketone salts (KS) (Evans et al., 2017; Rodger et al., 2017; O'Malley et al., 2017; Waldman et al., 2018), and the ketogenic compound 1,3-butanediol (BD) (Scott et al., 2019; Shaw et al., 2019) prior to and/or during an exercise challenge. One of the key metabolic consequences of ingesting exogenous ketone supplements is the elevation in circulating βHB, but we speculate that exercise performance is unlikely to be affected unless βHB concentrations exceed 1.0 mM (Evans, Cogan and Egan, 2017). To date, the only supplement to consistently exceed this threshold prior to an exercise challenge is the KME supplement (Cox et al., 2016; Evans & Egan, 2018). KS and KDE elevate βHB concentrations into the 0.3 to 0.6 mM range (Evans et al., 2017; Rodger et al., 2017; Leckey et al., 2017), and ingestion of BD elevates βHB concentrations into the 0.6 to 0.8 mM range (Scott et al., 2019; Shaw et al., 2019).

Specifically focusing on KME ingestion and exercise studies, ingestion of 573 $mg.kg^{-1}$ body mass of KME in the fasted state elevated βHB concentrations to ~2.0 mM 20 min after ingestion where it remained throughout 1 h cycling exercise at 75%W_{max} and a subsequent 30 min TT (Cox et al., 2016). In the fed state, ingestion of 750 $mg.kg^{-1}$ body mass of KME elevated βHB concentrations to >1.5 mM after 15 min of exercise, and ~2.6 mM by the end of 75 min of intermittent running followed by a short duration shuttle run to exhaustion

(Evans & Egan, 2018). In contrast to this previous work, plasma βHB concentrations in the present study were elevated to ~1.3 mM during the exercise protocol, which is lower than previously observed at the same 573 $mg.kg^{-1}$ body mass dose (Cox et al., 2016). This attenuated rise in plasma βHB concentrations is unsurprising given that ingestion of KME in the fasted states consistently elevates circulating βHB to >3.0 mM (Stubbs et al., 2017; Stubbs et al., 2018), whereas ingestion of KME in the postprandial state results in circulating βHB in the range from ~1.0 to 2.5 mM (Cox et al., 2016; Stubbs et al., 2017; Evans & Egan, 2018). For instance, ingestion of 395 $mg.kg^{-1}$ body mass in the fasted state produces peak βHB concentrations of ~3.0 mM but only ~2.0 mM in the fed state, a 33% reduction in C_{max} and coinciding with a 27% reduction in 4 h βHB AUC in resting participants (Stubbs et al., 2017). Given that our participants were fed a lower initial dose of KME of 287 $mg.kg^{-1}$ body mass, that this ingestion occurred in a postprandial state, and that exercise commenced 30 min later, it is not surprising that we observed lower βHB concentrations prior to and during exercise compared to previous work.

Although the present protocol achieved acute nutritional ketosis, a benefit to endurance performance was not observed. This finding is consistent with a number of studies that have failed to find a performance benefit of exogenous ketone supplements in various exercise models (Rodger et al., 2017; O'Malley et al., 2017; Leckey et al., 2017; Waldman et al., 2017; Evans & Egan, 2018). The variety of exogenous ketones supplements used, the large range of changes in circulating βHB produced, and a lack of consistency in the nutrients co-ingested and type of exercise challenge performed, make it difficult to make broad conclusions on the efficacy of these supplements. However, only one study to date has demonstrated a performance benefit with the ingestion of KME, which when co-ingested with CHO increased the distance covered in a 30 min cycling TT by ~2% (411 ± 162 m), when preceded by 1 h pre-load exercise at 75% W_{max} (Cox et al., 2016). The proposed

mechanism for this improvement in performance was a shift in the contribution to energy provision from substrate utilization of carbohydrate to fat, as demonstrated by reduction in glycolytic flux resulting in a 'sparing' of muscle glycogen, and a concomitant increase in intramuscular triglyceride utilization during exercise (Cox et al., 2016).

The mechanistic basis whereby elevated ketones reduce carbohydrate utilization during exercise is likely an attenuation of glycolytic flux via an inhibition of pyruvate dehydrogenase and phosphofructokinase by increases in NADH:NAD^+, acetyl-CoA:CoA, or citrate. A reduction in glycolytic flux has been proposed to explain the attenuated exercise-induced rise in plasma lactate observed in previous studies providing KME (Cox et al., 2016; Evans & Egan, 2018). This attenuation was ~50% during 60 min at 75%W_{max} and 30 min TT in trained cyclists (Cox et al., 2016), and ~10% to 30% during 75 min of intermittent running in team sport athletes (Evans & Egan, 2018). However, no differences in plasma lactate were observed between trials in the present study either during the pre-load or TT periods. The submaximal exercise intensity of ~65%$\dot{V}O_{2peak}$ employed was below lactate threshold for all participants, and therefore an intensity too low to observe an attenuation, if any, of the exercise-induced rise in plasma lactate. However, plasma βHB concentrations were elevated >1.0 mM before and at the cessation of the 10-km TT, yet no difference in plasma lactate was observed between trials.

Similarly, while a glucose-lowering effect of KME ingestion is well-documented whether ingested alone (Cox et al., 2016; Stubbs et al., 2017; Stubbs et al., 2018), or co-ingested with carbohydrate or protein (Cox et al., 2016; Stubbs et al., 2017; Myette-Côté et al., 2018; Vandoorne et al., 2017; Evans & Egan, 2018), we observed an attenuation in the rise in plasma glucose concentrations only at 30 min after ingestion of the first bolus of CHO+KME compared to CHO+PLA. This difference in plasma glucose between trials was absent during the submaximal exercise period and cessation of the 10-km TT. When effects

of KME ingestion on plasma glucose have been observed, the mechanism proposed has been an attenuation of hepatic gluconeogenesis and an increase in hepatic glucose uptake (Myette-Côté et al., 2018). Under certain conditions, elevated ketone body concentrations may have an insulinotropic action (Balasse & Fery, 1989) but is not always observed (Nair et al., 1988; Mikkelsen et al., 2015). When co-ingested with carbohydrate and/or protein, the effect of exogenous ketones to attenuate postprandial glycemia occurs despite similar circulating insulin concentrations between conditions (Cox et al., 2016; Vandoorne et al., 2017; Myette-Côté et al., 2018).

We propose that the lack of differences between trials for plasma glucose and lactate in contrast to previous work suggests that the nature of the exercise challenge, or the degree of nutritional ketosis are key determinants of the metabolic effects of exogenous ketone supplements during exercise. While plasma βHB concentrations were elevated to ~1.3 mM at the cessation of the 10-km TT, concentrations were ~1.2 mM lower than observed in previous work demonstrating effects on plasma glucose and lactate during exercise (Evans and Egan, 2018; Cox et al., 2016), The lower plasma βHB concentrations are a consequence of the aforementioned particulars of the dosing and feeding strategy, and future research should be cognizant of these issues when designing study protocols.

The brain is the primary site of ketone utilization under conditions of low carbohydrate availability (Owen et al., 1967). Elevated βHB concentrations are associated with a neuroprotective role in non-exercise contexts (Ari et al., 2016; Kovacs et al., 2017; Svart et al., 2018), and short-term (5 d) feeding of a diet was supplemented with KME improved performance of rats in a radial maze task by 38%, and improved decision-making during the test (Murray et al., 2016). Moreover, in our previous work, acute ingestion of KME preserved cognitive performance, measured by the number of incorrect responses to a multi-tasking test (Evans & Egan, 2018). This test was performed at the cessation of a short

duration intermittent run to exhaustion proceeding the Loughborough Intermittent Shuttle Test (LIST), a variable intensity running protocol that mimics soccer match-play (Nicholas et al., 2000). In contrast to previous results, we observed no difference in cognitive performance with the addition of KME in the present study. The specifics of the exercise challenge may play a role in these divergent findings. The LIST is a mentally-demanding task that requires participants to be aware of current and subsequent running speeds for 75 min. Mental fatigue has a negative impact on aspects of cognitive performance, including altered attentional focus (Boksem et al., 2005) and slower and less accurate reaction times (Boksem et al., 2006), suggesting that the more mentally demanding the task the larger deficit should be evident in cognitive performance. In the present study, we observed no decline in cognitive performance in either condition. The absence of decline is important to note because in our previous work, it was a preservation of cognitive performance with KME, not an absolute improvement (Evans and Egan, 2018). These results suggest the exercise challenge presently employed was not sufficiently mentally-demanding to negatively impact reaction time or executive function, and therefore, potential benefits were unlikely to be observed.

Concerns have been raised about the practical use of exogenous ketone supplements by athletes due to the high rates of occurrence of GI distress in previous work using BD (Shaw et al., 2019), KS (Evans et al., 2017; Fischer et al., 2018), KDE (Leckey et al., 2017), and KME (Evans & Egan, 2018). However, in the present study, incidences of GI distress were similar between conditions, and this is consistent with previous work using KME (Cox et al., 2016). Typically, rates of occurrence of GI distress are higher with exogenous ketone ingestion occur at a higher rate with increasing doses (Clarke et al., 2012; Evans et al., 2017; Evans & Egan, 2018). Importantly, no participants nominated GI distress as a distraction or detriment to performance during CHO+KME trials.

In conclusion, the addition of a commercially-available ketone monoester supplement to a carbohydrate-based fuelling strategy prior to and during exercise did not improve performance in a self-paced, treadmill-based 10-km TT. Ingestion of the ketone monoester attenuated the rise in plasma glucose prior to exercise but concentrations were similar between trials thereafter, and no effect on the increase in plasma lactate concentrations during the 10-km TT was observed. Moreover, no differences between trials were observed for a range of physiological responses, and assessments of cognitive performance. Future research should evaluate different dosing strategies and exercise models to elucidate whether a threshold of plasma βHB concentration must be exceeded in order to exert performance benefits, and in which exercise contexts these benefits, if any, might be realized.

Chapter 6

Main research findings

When starting this body of work in winter of 2015, there was a dearth of published research on exogenous ketones and performance despite their use in professional sport being rumoured in that year (Abraham, 2015). Shortly thereafter, the first published report demonstrating pleiotropic effects on exercise metabolism and a meaningful ergogenic effect in elite time-trial performance emerged (Cox et al., 2016). Three years later, the area continues to grow in scope. The aim of this thesis was therefore to elucidate, if any, the metabolic effects of acute exogenous ketone ingestion prior to exercise and their ergogenic potential in other exercise contexts. The studies and their key findings are summarised as follows:

Study 1

Ingestion of βHB ketone salts (KetoCaNa, KetoSports, United States) in two boluses of 0.38 $g.kg^{-1}$ body mass prior to exercise elevated plasma βHB concentrations to ~0.4-0.5 mM. Ingestion of the ketone salts decreased blood glucose by ~10% but had no effect on blood lactate concentrations versus a water control. These results agree with other work demonstrating a decrease in blood glucose even when βHB concentrations are <1.0 mM (O'Malley et al., 2017), but this does not always occur (Waldman et al., 2017; Rodger et al., 2017).

Study 2

Ingestion of 750 $mg.kg^{-1}$ body mass of a βHB monoester (KME) (KE4, KetoneAid, United States) had no effect on 15 m sprint times during 75 min of soccer simulated intermittent activity. Intermittent running time to exhaustion may have been impaired with βHB monoester ingestion. A novel finding of this work was the preservation of cognitive function with βHB monoester ingestion, measured by incorrect decisions during a decision-making test but no difference was observed in reaction time or sustained attention. This work

contrasts with the seminal report on the ergogenic potential of exogenous ketones (Cox et al., 2016) where an ergogenic effect of a βHB monoester was observed in a 30-min maximum distance cycling time trial. However, the metabolic effects of elevated βHB concentrations in this study were a decrease in blood glucose and lactate concentrations, which are in agreement with other works (Cox et al., 2016; Stubbs et al., 2017; Leckey et al., 2017; Myette-Côte et al., 2018).

Study 3

Ingestion of 573 $mg.kg^{-1}$ body mass of the (R)-3-hydroxybutyl (R)-3-hydroxybutyrate ketone monoester (HVMN Ketone, HVMN, United States) had no effect on a 10-km self-paced time trial following a 60-min pre-load at 65% $\dot{V}O_{2peak}$. Cognitive performance improved to a similar extent in various measures in both conditions. These data contrast with previous results investigating the ergogenic potential of KME in endurance exercise (Cox et al., 2016) and in cognitive performance (Evans et al., 2018). We previously observed a preservation of cognitive performance with KME ingestion, but this is not to be confused with an improvement in cognitive performance. Both conditions in this study improved in several measures of cognitive performance in a similar manner.

Metabolic response to ketone salt ingestion during exercise

Acute ingestion of two servings ketone salts (KS) in the hour prior to exercise elevates plasma βHB concentrations to ~0.44 ± 0.27 mM (Evans et al., 2018), while one serving KS elevates whole blood βHB concentrations between 0.5-0.8 mM (O'Malley et al., 2017; Rodger et al., 2017; Waldman et al., 2018), when measured by handheld point-of-care monitors. This modest elevation in βHB is accompanied by an ~10% decrease in plasma glucose during submaximal exercise (O'Malley et al., 2017; Evans et al., 2017). During submaximal exercise, plasma lactate is not affected by acute KS ingestion (Rodger et al.,

2017; O'Malley et al., 2017; Waldman et al., 2018; Evans et al., 2018). RER findings are divergent, both raising (Evans et al., 2018) and lowering (O'Malley et al., 2017) the RER, which may be accounted for by the difference in serving size and training status of participants. Doubling the recommended dosage of KS was accompanied by a high incidence of gastrointestinal distress during exercise, namely nausea, diarrhoea, vomiting and light headedness, ostensibly because of the hyperosmotic salt load delivered (Evans et al., 2018).

Higher HR values have been observed in response to ketone salt ingestion during exercise (Evans et al., 2018) and βHB infusions at rest (Gormsen et al., 2017; Svart et al., 2018), but this is not always observed during exercise (Waldman et al., 2018).

Metabolic response to ketone ester ingestion during exercise

In one of a series of experiments, ingestion of 573 $mg.kg^{-1}$ body mass (R)-3-hydroxybutyl (R)-3-hydroxybutyrate ketone monoester (KME) prior to 45 min cycling at 40% and 75% peak power output elevated βHB concentrations to ~4.0 mM and ~3.0 mM respectively and βHB contributed 18% and 16% towards total oxygen consumption. The exercise induced rise in plasma lactate was attenuated by ~50% during 1 h cycling at 75% W_{max} compared to isocaloric carbohydrate ingestion (Cox et al., 2016). Similarly, in study 2 of this thesis, ingestion of KME resulted in elevated plasma βHB concentrations of ~1.0-3.0 mM, reduced plasma lactate and glucose concentrations in team sport athletes throughout a simulated soccer protocol (Evans & Egan, 2018). In study 3 of this thesis, we observed a reduction in plasma glucose 30 min after ingestion of KME, thereafter concentrations were similar between KME and placebo conditions throughout a 1 h pre-load at 65% $\dot{V}O_{2peak}$ and after a 10-km self-paced treadmill based time trial, but with no differences in plasma lactate concentrations.

The glucose-lowering effect of KME and KDE when ingested prior to exercise is well-demonstrated (Cox et al., 2016; Leckey et al., 2017; Vandoorne et al., 2017; Myette-Côté et al., 2018; Evans & Egan, 2018). This effect is most likely explained by reduced hepatic gluconeogenesis (Mikkelsen et al., 2015) and occurs independently of differences in circulating insulin between conditions where KME is present or absent with carbohydrate or carbohydrate-protein ingestion (Vandoorne et al., 2017; Myette-Côté et al., 2018; Vandoorne).

KME and KDE ingestion prior to exercise attenuates the exercise-induced increase in plasma lactate during endurance and intermittent activity (Cox et al., 2016, Leckey et al., 2017; Evans & Egan, 2018), but in study 3 on this thesis did not occur during a 60-min pre-load at 65% $\dot{V}O_{2peak}$ or after the 10 km time-trial in a population of trained endurance runners following KME ingestion. These differences are explained by the intensity of the pre-load, which was below each runner's lactate threshold and did not elevate plasma lactate in either condition. One of the main proposed benefits of exogenous ketones is a 'glycogen sparing' mechanism that will confer an advantage during periods of competition that are high intensity in nature and carbohydrate-dependent (Pinckaers et al., 2017; Evans et al., 2017). Carbohydrate utilisation is reduced during submaximal exercise with KME ingestion in the fasted state (Cox et al., 2016), and it remains to be seen whether this sparing of carbohydrate can be overcome later in exercise or whether it will impair performance if the exercise duration is long enough. When compared to the lack of effect of KS ingestion on plasma lactate, βHB concentrations between ~1.0 to 2.0 mM may be required to elicit an attenuation in plasma lactate concentrations.

Effect of acute ingestion of a βHB ketone monoester on physical performance

Ingestion of 573 $mk.kg^{-1}$ body mass KME improved 30-min max distance cycling time trial performance by ~2% (411 ± 162 m), following a 1 h pre-load at 75% $W_{max.}$ The

drinks consumed in these trials were isocaloric, meaning performance improved despite receiving less carbohydrate during the KME trial (Cox et al., 2016). Since then, only two reports on the physical performance effects of acute ketone ester ingestion have been published (Leckey et al., 2017; Evans & Egan, 2018). Only the latter (study 2 of this thesis) utilised a KME and therefore is discussed in detail for contrast with the work of Cox et al. (2016). Study 3 of this thesis will add to literature in this area.

Ingestion of 750 $mg.kg^{-1}$ body mass KME ingested 20-min before exercise and at 30- and 60-min of soccer simulated intermittent activity divided in three aliquots (50:25:25) had no effect on 15 m sprint times during a 75 min simulated soccer protocol or subsequent run to fatigue time (KME, 229±72 s; PLA 267±96 s; p=0.126; ES=0.45 small) in team sport athletes (Evans & Egan, 2018).

The divergent findings in relation to performance between study 2 and the original KME work (Cox et al., 2016) may be explained by the training level of the participants. KME ingestion elevated plasma βHB to >1.5 mM at start of exercise and reached ~2.6 mM at the end of exercise, and reduced plasma lactate and glucose concentrations during exercise (Evans & Egan, 2018). The concentrations are similar to those achieved in endurance trained cyclists, however, KB are transported across the skeletal muscle membrane by monocarboxylate transports (MCT). MCT concentrations are highest in type 1 muscle fibres, and are increased in response to endurance training (Bonen, 2001; Thomas et al., 2012). This would suggest endurance athletes would have a higher capacity to extract KB from the blood and oxidise them as a substrate.

In trained runners, ingestion of 573 $mg.kg^{-1}$ body mass KME did not improve 10 km treadmill based time trial performance. KME was split into three aliquots (50:25:25) and ingested 30 min prior to and at 20 and 40 min of a 1 h pre load at 65% $\dot{V}O_{2peak.}$ These findings contrast to the ergogenic effect observed in cyclists with a similar dosing and timing

strategy (Cox et al., 2016). This may be accounted for in the different endurance modalities employed in the studies (cycling vs. running). On the rationale of an ergogenic benefit due to a 'glycogen sparing' effect, it is possible that either the intensity of the pre-load was not sufficient to elicit an substantial depletion of muscle glycogen, or the duration of the performance test (35-45 min) was not long enough to be limited by muscle glycogen availability. However, the previous report utilised a 30 min maximal distance cycling time trial so this cannot account for the difference between studies.

Furthermore, the central tenet is that the combination of fuel sparing and improved energetic efficiency during acute nutritional ketosis confers performance benefits (Cox et al. 2016). Alterations in fuel selection during steady-state exercise have been demonstrated, which indicate reduced glycolytic flux, sparing of CHO and increased contribution of IMTG and βHB to energy provision (Cox et al., 2016). Whether this sparing of CHO, in fact, manifests as impaired CHO utilisation remains to be determined. The mechanistic basis for CHO sparing by exogenous ketones is presently proposed as inhibition of glycolytic flux via inhibition of PDH and PFK by increases in NADH:NAD^+, acetyl-CoA:CoA ratio or citrate. In theory, this could be problematic for sports that rely heavily on contributions from glycolytic pathways, or a range of sports that are intermittent and/or require periods of high intensity 'bursts' on a moderate intensity background. However, the use of 'sparing' or 'impairing' of glycogen may be a misnomer, as both refer to the downregulation of carbohydrate metabolism via the same mechanism. In the current work, glycogen sparing refers to a downregulation of carbohydrate metabolism early in exercise, alongside an elevation in circulating βHB due to the ingestion of exogenous ketones, which can then be utilised later in the exercise challenge. If this downregulation of carbohydrate metabolism cannot be overcome later in exercise, resulting in an impairment of carbohydrate oxidation,

this would be termed glycogen impairing and likely lead to a performance decrement in sporting events that require a high rate of ATP turnover.

Cognitive performance after exercise after ingestion of a βHB ketone monoester

Cognitive performance after acute exogenous KS or KME ingestion is not well described in humans with only two recent reports in the published literature (Waldman et al., 2018; Evans & Egan, 2018) and study 3 will add to this area. Ingestion of 750 $mg.kg^{-1}$ body mass KME before and during exercise preserved executive function compared to a placebo drink, measured by the number of incorrect responses to a multi-tasking test. KME ingestion had no effect on reaction time or sustained attention, measured by latency of response and correct/incorrect responses. Cognitive tasks were performed immediately after a 75 min simulated soccer protocol and subsequent 4-6 min run to exhaustion (Evans & Egan, 2018). Following acute ingestion of one serving KS, participants completed a 5-min reaction test during their warm up and immediately following 4x15 s anaerobic Wingate sprints on a cycle ergometer. No effect on reaction time was observed after the anaerobic activity despite a higher fatigue index in the KS condition (KET: 32.3 ± 13.9 $W.s^{-1}$; PLA: 29.2 ± 12.6 $W.s^{-1}$), however a learning effect is noted by the authors for hits and misses on the same test (Waldman et al., 2018). These studies used different forms of exogenous ketones, dosing strategies and performance tests. These protocols may not have been long enough to cause the aforementioned reduction in whole body energy stores or dehydration to cause substantial decline in cognitive function (Tomporowski, 2003), and would seemingly agree that short exercise has little impact on cognitive function and exercise lasting <90 min exerts a selective influence on cognitive ability.

In study 3 of this thesis, in contrast to previous results in study 2, we observed no difference in cognitive performance with the addition of KME to carbohydrate compared to

placebo ingestion. The specifics of the exercise challenge may play a role in these divergent findings. The Loughborough Intermittent Shuttle Test used in study 2 is a mentally-demanding task that requires participants to be aware of current and subsequent running speeds for 75-min. Mental fatigue has a negative impact on aspects of cognitive performance, including altered attentional focus (Boksem et al., 2005) and slower and less accurate reaction times (Boksem et al., 2006), suggesting that the more mentally demanding the task the larger deficit should be evident in cognitive performance. In study 3, we observed no decline in cognitive performance in either condition. The absence of decline is important to note because in our previous work, it was a preservation of cognitive performance with KME, not an absolute improvement (Evans & Egan, 2018). These results suggest the exercise challenge employed and the degree of mental challenge are important factors when evaluating the effect on cognitive performance

Table 6. 1 Summary of the effects of ketogenic supplements on physical and cognitive performance.

Paper	Exercise challenge	Study design	Exogenous ketone supplement	Dose of ketone supplement	βHB concentrations	Effects on physical performance	Effects on cognitive performance
Cox et al., (2016)	1 h steady state cycling (75% W_{max}) 30 min maximal distance cycling time trial	βHB monoester plus carbohydrate vs isocaloric carbohydrate	(R)-3-hydroxybutyrate-(R)- 1,3-butanediol monoester	573 $mg.kg^{-1}$ body mass (2:1:1)	2.0-2.5 mM	Time trial performance improved with addition of βHB monoester by approx. 2% (411 ± 162 m)	N/A
O'Malley et al., (2017)	3x5 min cycling (30, 60, 90% VT) 150 kJ time trial (approx. 10km)	βHB salt vs flavour matched acaloric placebo	βHB salt	0.3 $g.kg^{-1}$ body mass βHB salt	0.8 mM	Time trial performance impaired with βHB salt βHB salt: 711 ± 137 s Placebo: 665 ± 120 s	N/A
Rodger et al., (2017)	90 min cycling (80% VT_2) 4 min maximal cycling	βHB salt vs flavour matched acaloric placebo	βHB salt	2 servings (11.7 βHB per serving)	0.6 mM	No difference in maximal or average wattage between	N/A

	performance task					conditions	
Waldman et al., (2017)	Four maximal 15 s cycling sprint trials (4 min active recovery)	βHB salt vs flavour matched acaloric placebo	βHB salt	1 serving (11.38 g βHB)	0.5 mM	Higher fatigue index during 15 s sprints in βHB salt condition. No effect of condition on mean power, peak power or total work performed.	No effect of βHB salt on average or slowest response time during a reaction time task
Leckey et al., (2017)	4x5 min warm up 31.2 km cycling time trial	AcAc diester vs taste matched placebo	R,S-1,3-butanediol acetoacetate diester.	500 $mg.kg^{-1}$ body mass split in to two boluses	0.4 mM	Time to complete time trial approx. 2% longer with AcAc diester vs. placebo ingestion (58 s).	N/A
Evans and Egan (2018)	75 min intermittent running Intermittent run to exhaustion	βHB monoester plus carbohydrate vs isocaloric carbohydrate	(R)-3-hydroxybutyrate-(R)- 1,3-butanediol monoester	750 $mg.kg^{-1}$ body mass	1.0 - 2.6 mM	No effect on 15 m sprint times during 75 min intermittent running My have impaired short duration intermittent	No effect of condition on tests of reaction time or sustained attention. Addition of βHB

						running to exhaustion	monoester may have preserved decision making
Evans et al., (2019)	1 h treadmill running (65% VO_{2peak}) Self paced treadmill based 10 km time trial	βHB monoester plus carbohydrate vs isocaloric carbohydrate	(R)-3-hydroxybutyrate-(R)- 1,3-butanediol monoester	573 $mg.kg^{-1}$ body mass	1.6 mM	No effect on self paced 10 km time trial performance	No effect of condition on tests of reaction time or decision making

Emerging issues and future directions for research in exogenous ketone supplementation in athletic performance

Considering the recent emergence as exogenous ketone supplements as a method to induce acute nutritional ketosis and alter the metabolic response to exercise, more research on their effects on physical and cognitive performance needs to be undertaken before any statements can be made on the ergogenic potential for athletes and active adults. There remains only report of an ergogenic effect of exogenous ketones (Cox et al. 2016). Reports on various forms of exogenous ketones have reported either no effect (Rodger et al. 2017, Waldman et al. 2017, Evans and Egan. 2018, Study 3) or an impairment in physical performance (Leckey et al. 2017, O'Malley et al. 2017).

As with any ergogenic aid or nutrition strategy, optimising dosing strategies including quantity and timing will be important. Given the saturation kinetics of KB oxidation by skeletal muscle and curvilinear relationship between oxidation and plasma concentrations, it is likely that there is an optimal range for performance benefits. At present, we speculate that this exists between 1 and 3 mM βHB. As with many ergogenic acids, more is unlikely to be better and may even be deleterious given the potential for acidosis at higher KB concentrations, and aforementioned gastrointestinal distress and other side-effects sometimes observed with KS, KME and KDE, so careful consideration should be given to these issues.

KME, KDE and KS are proposed as performance-enhancing dietary supplements and are available for purchase commercially, with the exception of KDE, which is only available for research purposes. Performance-enhancing dietary supplements pose a greater challenge than other forms of dietary supplements due to the scarcity of quality research applicable to the elite athlete (Maughan et al., 2018). Future studies in exogenous ketone supplementation should be cognisant of several of the following important aspects of performance science

research to make the largest impact possible (Maughan et al., 2018; Burke & Peeling, 2018). Studies should be conducted in double-blinded design where participants are randomised to a control or experimental group or in a crossover design where participants receive both treatments. Researchers should aim for adequate sample sizes and appropriate participant characteristics to gain statistical power, should mimic conditions of real-life events, standardise pre-trial preparation across trials (exercise/diet/caffeine ingestion etc.) and environmental factors during each trial (temperature, encouragement), use a tested verified non-contaminated substance with an appropriate protocol (dosage/timing) and use a reliable and valid performance test (Maughan et al., 2018; Burke & Peeling, 2018). Studies 2 and 3 of this thesis have aimed to satisfy these recommendations.

Standardising of reporting methodologies specifically related to exogenous ketone supplementation will be important to elucidate that may benefit from supplementation and are outlined below.

βHB measurement*:* Verification that a potential performance-enhancing supplement was ingested and elicited a biological response should be undertaken in sports performance research (Maughan et al., 2018). Ingestion of KS, KME and KDE elevates blood/plasma βHB concentrations and should be used to measure responses in experimental and placebo conditions. Laboratory assays using reagent and colorimetric assays (Daytona; Sigma Aldrich) should be considered the gold standard for measurement of plasma βHB concentrations to standardise interpretation of results. Handheld monitors (Precision Neo, etc) measure whole blood βHB and overestimated βHB concentrations by ~1.0 mM during a 31.2 km time trial in cyclists compared to βHB measured in serum samples (Leckey et al., 2017). If using handheld monitors, overestimation should be clearly mentioned and should be reported as whole blood βHB concentrations. The use of handheld monitors raises an issue with the blinding of a performance study, as they allow immediate measurement of whole blood βHB

concentrations. In a performance study utilising crossover design comparing exogenous ketones to a carbohydrate fed condition, βHB concentrations will remain at baseline in the carbohydrate condition and will be elevated in the ketone condition, de-blinding the study. If using a handheld monitor, a researcher who is not involved in the performance testing (encouragement etc.) should analyse whole blood samples for βHB concentrations and ensure that the participant is not aware of the concentrations in order to ensure that the experiment remains blinded.

Recovery*:* KB have an anti-catabolic effect (Thomson & Wu, 1991), attenuating leucine oxidation, and increasing MPS by 10% when βHB is elevated to ~ 2.0 mM (Nair et al., 1988). Work utilising a practically-relevant glycogen-depleting protocol and optimal carbohydrate and protein-based recovery strategies will elucidate whether the addition of exogenous ketones confers any benefit to glycogen re-synthesis and MPS. Recovery from exercise, in terms of glycogen re-synthesis becomes more important when a short amount of time is available between bouts of exercise (i.e. multiple heats). Recently, infusion of ketone bodies produced a robust anticatabolic response under an acute inflammatory condition insult provided by lipopolysaccharide endotoxin, reducing phenylalanine efflux by 70% in fasting conditions over the course of 6 h compared to saline and free fatty acids (Thomsen et al., 2018). Maintenance of lean mass during calorie restriction and weight loss, which may be applicable to weight dependent sports or during periods of intensive training is of interest. To our knowledge, there is no work investigating the anticatabolic effects of ketone bodies in skeletal muscle in combination with amino acids. However, ingestion of 0.5 $mg.kg^{-1}$ body mass KME provided alongside carbohydrate and protein during a 5 h recovery period from a glycogen-depleting protocol resulted in higher mTORC1 activation via downstream signalling compared to a placebo carbohydrate and protein drink (Vandoorne et al., 2017). If the addition of ketone bodies to amino acids produces a greater net muscle protein balance,

either by increasing MPS or reducing muscle protein breakdown, it may represent a novel recovery paradigm worth investigating in athletes.

***Exogenous ketone dose**:* KME, KDE and KS dosages are reported using a variety of methods: $mg.kg^{-1}$ body mass (Cox et al., 2016; Leckey et al., 2017; Evans et al., 2018), $mM.kg^{-1}$ body mass (Stubbs et al., 2017), $kcal.kg^{-1}$ (Stubbs et al., 2018) and $mL.kg^{-1}$ (Holdsworth et al., 2017; Myette-Côté et al., 2018) and $g.kg^{-1}.h^{-1}$ (Vandoorne et al., 2017). Researchers and practitioners require a standardised reporting method to inform their own work and avoid misinterpretations. A standardised measurement of $mg.kg^{-1}$ body mass total supplement is recommended. A salient issue with KS ingestion is the associated gastrointestinal distress experienced with higher dosages (Evans et al, 2017). Despite receiving double the manufacturers recommended dosage, βHB was elevated to ~0.4-0.5 mM during a graded exercise session, which is well below the ~1.0 mM threshold needed to have a meaningful contribution to fuel provision and the hypothesised level to enhance performance (Cox et al., 2016; Evans et al., 2017). Under fed conditions, KME dose >573 $mg.kg^{-1}$ body mass may be required to elevate βHB to 1.0-3.0 mM and elicit an ergogenic effect.

***Substrate oxidation**:* Traditional equations used to calculate substrate oxidation assume negligible contributions from non-carbohydrate and fat sources, including KB (Frayn, 1983). KME ingestion increases the contribution of βHB to total oxygen consumptions to 16% to 18%, calculated using novel and possibly flawed methods of estimation (Cox et al., 2016). Therefore, use of traditional methods for calculating substrate oxidation are unsuitable during acute nutritional ketosis. Careful interpretation of RER during exercise is needed, as the stoichiometry of AcAc, the final step in KB oxidation, is 1.00, similar to that of carbohydrate (Frayn, 1983). A recent report on a shift from carbohydrate to fat oxidation during an incremental steady state protocol and subsequent time trial using traditional equations likely

do not reflect the true substrate oxidation proportions during the exercise challenge (O'Malley et al., 2017). Reporting of RER is likely to be all that is valid in reporting indirect calorimetry data unless the methods of Frayn (1983) are employed, but these too rely on assumptions that might not be valid in the context of acute nutritional ketosis.

Product racemity*:* Commercially-available KS provide a racemic mixture of βHB, i.e. containing both the D- and L- enantiomers of βHB (also designated R- and S-, respectively), whereas various βHB assays only determine D-βHB concentrations. Ingestion of a commercially available KS (KetoForce, KetoSports, United States) elevates L-βHB to ~2.0 mM (Stubbs et al., 2017). However, L-βHB is not a substrate for mitochondrial BDH a key ketolytic enzyme and is not metabolised to AcAc, and is therefore not a substrate for skeletal muscle metabolism (Scofield et al., 1982). If possible, purity analysis defined as the D-βHB content of the supplement, or L-βHB analysis should be undertaken to account for differences in product racemity.

Storage of samples*:* βHB is a very stable metabolite in plasma and whole blood samples, allowing for measurement up to 7 days after sample collection when stored at 4 °C, or for longer periods when stored at -80 °C (Custer et al., 2001) Degradation of AcAc in plasma samples was thought to be rapid, with guidelines suggesting measurement must take within 24 h of sample collection or stored for no longer than 3 days at -20 °C. This was based on data showing 80% and 30% of AcAc was lost from plasma samples after 3 d storage at room temperature and -20 °C, respectively (Price et al., 1977; Stubbs et al., 2018). However, degradation of AcAc in plasma is reduced when stored at -80 °C, with only 14% lost over 40 days of storage (Fritzsche et al., 2001). Moreover, AcAc is stable when de-proteinised with perchloric acid and stored at -80 °C for 60 days, and is more stable in plasma than whole blood samples. De-proteinisation ensures removal of 3-hydroxutyrate dehydrogenase, meaning that AcAc is not being reduced to βHB (McNeil et al., 2014). A comparison

between non de-proteinised and de-proteinised samples was not undertaken in this latest study (McNeil et al., 2014), but no difference in AcAc concentrations were noted when samples were left proteinised and non-de-proteinised prior to analysis in a previous study (Galán et al., 2001). Analysis of AcAc concentrations should be undertaken in studies using ketogenic supplements as AcAc can be reduced to βHB in collected samples. Samples for AcAc analysis should be immediately stored and measurement validity may be improved by de-proteinisation with perchloric acid.

Concluding remarks

In conclusion, although data are preliminary and somewhat conflicting, acute nutritional ketosis achieved by ingestion of exogenous ketone supplements has certainly the potential to alter fuel selection and the metabolic response during exercise, but the evidence to confer performance benefits remains sparse. Benefits to performance, if any, are most likely to be observed in trained individuals who have a greater capacity to uptake and oxidise KB during exercise because of their aerobic training base and the consequent ketolytic adaptations in skeletal muscle. Additionally, a strong physiological basis, albeit presently circumstantial, exists that suggests potential benefits for supporting training adaptation and recovery. While much work remains to be performed, particularly in relation to sport-specific strategies, this promises to be an exciting topic for scientists, practitioners and athletes alike for the coming years.

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Appendices

Appendix A: Published Journal of Physiology article

The Journal of Physiology

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TOPICAL REVIEW

Metabolism of ketone bodies during exercise and training: physiological basis for exogenous supplementation

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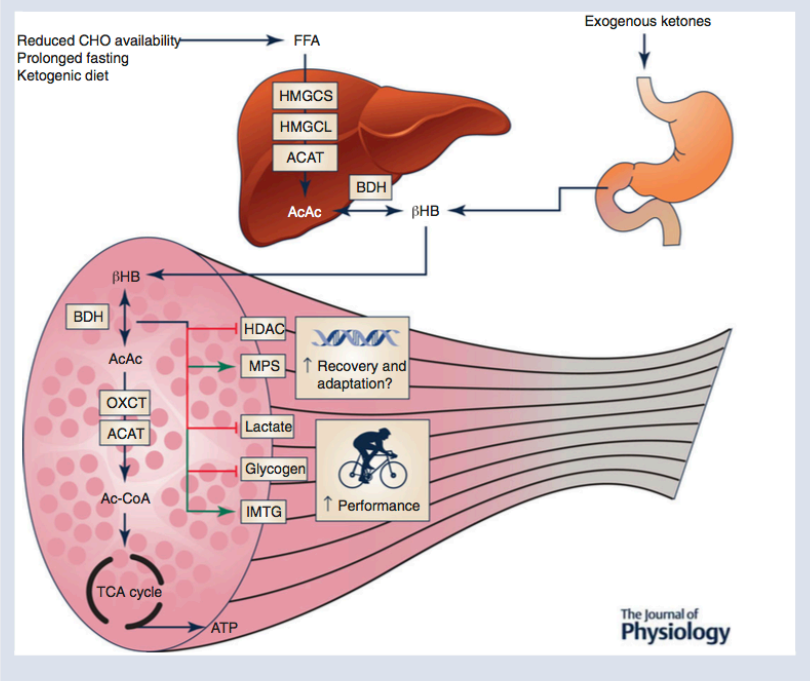

Mark Evans and **Karl Cogan** are graduate students at the Institute for Sport and Health, University College Dublin, Ireland. Mark received his MSc in Sport Nutrition from Liverpool John Moores University in 2015. Karl received his MSc in Biotechnology from University College Dublin in 2013. Their research explores optimising nutrition strategies for performance and recovery in athletes with specific interest in ketone bodies and protein hydrolysates, respectively. **Brendan Egan PhD** is Senior Lecturer in Sport and Exercise Physiology at Dublin City University's School of Health and Human Performance, and Visiting Associate Professor at University College Dublin. His research group investigates the molecular regulation of skeletal muscle function, adaptation and performance across the life course with special interest in the synergy between nutrition and exercise interventions ranging from athletes to older adults. All three authors are accomplished sportsmen in their own right, and currently involved in the provision of sports science support to team sport athletes.

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Abstract Optimising training and performance through nutrition strategies is central to supporting elite sportspeople, much of which has focused on manipulating the relative intake of carbohydrate and fat and their contributions as fuels for energy provision. The ketone bodies, namely acetoacetate, acetone and β-hydroxybutyrate (βHB), are produced in the liver during conditions of reduced carbohydrate availability and serve as an alternative fuel source for peripheral tissues including brain, heart and skeletal muscle. Ketone bodies are oxidised as a fuel source during exercise, are markedly elevated during the post-exercise recovery period, and the ability to utilise ketone bodies is higher in exercise-trained skeletal muscle. The metabolic actions of ketone bodies can alter fuel selection through attenuating glucose utilisation in peripheral tissues, anti-lipolytic effects on adipose tissue, and attenuation of proteolysis in skeletal muscle. Moreover, ketone bodies can act as signalling metabolites, with βHB acting as an inhibitor of histone deacetylases, an important regulator of the adaptive response to exercise in skeletal muscle. Recent development of ketone esters facilitates acute ingestion of βHB that results in nutritional ketosis without necessitating restrictive dietary practices. Initial reports suggest this strategy alters the metabolic response to exercise and improves exercise performance, while other lines of evidence suggest roles in recovery from exercise. The present review focuses on the physiology of ketone bodies during and after exercise and in response to training, with specific interest in exploring the physiological basis for exogenous ketone supplementation and potential benefits for performance and recovery in athletes.

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Abstract figure legend Acetoacetate (AcAc) and β-hydroxybutyrate (βHB) are ketone bodies produced in hepatic mitochondria during conditions of reduced carbohydrate availability and serve as an alternative fuel source for peripheral tissues including skeletal muscle. Elevations in βHB can result from endogenous production i.e. ketogenesis, but also by ingestion of exogenous ketone supplements such as ketone salts or ketone esters. Ketogenesis from free fatty acids (FFA) involves sequential reactions of Ac-CoA acetyltransferase (ACAT), hydroxymethylglutaryl CoA synthase (HMGCS), and hydroxymethylglutary-CoA lyase (HMGCL). The end product of ketogenesis is AcAc, the majority of which is reduced to βHB by 3-hydroxybutyrate dehydrogenase (BDH) before entering the circulation. Upon uptake into peripheral tissues, βHB is oxidised to AcAc. Reactions of succinyl-CoA:3-oxoacid CoA transferase (OXCT) and ACAT ultimately produce acetyl CoA (Ac-CoA), which enters the TCA cycle for ATP synthesis. The metabolic actions of βHB include altered fuel selection during exercise through attenuating glycogen utilisation, lowering lactate production and increasing reliance on intramuscular triglyceride (IMTG). Additionally, βHB may regulate adaptive processes in skeletal muscle by acting as a signalling metabolite inhibiting histone deacetylases (HDAC), or through positive effects on muscle protein synthesis (MPS). Ketone ester supplements facilitate acute ingestion of βHB resulting in nutritional ketosis, which, through these mechanisms, may alter exercise metabolism, improve exercise performance, and influence recovery and the adaptive response to exercise.

Abbreviations AcAc, acetoacetate; AcAc-CoA, acetoacetyl CoA; ACAT, acetyl-CoA acetyltransferase; βHB, β-hydroxybutyrate; BDH, 3-hydroxybutyrate dehydrogenase; CHO, carbohydrate; CPT1, carnitine palmitoyltransferase; FFA, free-fatty acid; HDAC, histone deacetylase; HMG-CoA, hydroxymethylglutaryl-CoA; HMGCL, HMG-CoA lyase; HMGCS, HMG CoA synthase; KB, ketone body; KE, (*R*)-3-hydroxybutyl (*R*)-3-hydroxybutyrate ketone monoester; MCT, monocarboxylate transporter; OXCT, succinyl-CoA:3-oxoacid CoA transferase; PDH, pyruvate dehydrogenase; PEK, post-exercise ketosis; PFK, phosphofructokinase; PGC-1, peroxisome proliferator-activated receptor gamma coactivator 1; SLC, solute ligand carrier; TCA, tricarboxylic acid.

Introduction

Over the past century, exercise physiologists have appreciated the role of carbohydrate (CHO) and fat in energy provision to exercising skeletal muscle. Much of the work examining the metabolic response to exercise and the impact of exercise on metabolic regulation and adaptive responses to training has focused on the relative contribution of these fuels (Egan & Zierath, 2013). Optimising training and nutrition strategies by manipulating the relative intakes of these macronutrients is central to supporting elite sports performance (Cermak & van Loon, 2013; Bartlett *et al.* 2015; Burke, 2015).

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An alternative fuel source to CHO and fat are ketone bodies (KBs), namely acetoacetate (AcAc), acetone, and β-hydroxybutyrate (βHB), which are produced in the liver during physiological states and nutritional manipulations that result in reduced CHO availability, most commonly during prolonged fasting, starvation, and ketogenic (very low CHO (~5%), low protein (~15%), high fat (~80%)) diets (Robinson & Williamson, 1980; Laffel, 1999). This relative glucose deprivation and concomitant elevation in circulating free-fatty acids (FFAs) results in the production of KBs to replace glucose as the primary fuel for peripheral tissues such as the brain, heart and skeletal muscle in these states.

Aside from a role as an alternative fuel source, KBs exert a range of metabolic effects including attenuating glucose utilisation in peripheral tissues, anti-lipolytic effects on adipose tissue, and potential attenuation of proteolysis in skeletal muscle (Robinson & Williamson, 1980). KBs are utilised by working muscle during exercise (Fery & Balasse, 1986, 1988), and the capacity to take up and oxidise KBs during exercise is higher in exercise-trained skeletal muscle (Winder *et al.* 1975). Despite these observations, in addition to a glucose sparing action (Maizels *et al.* 1977) and potential to lower the exercise-induced rise in plasma [lactate] (Fery & Balasse, 1988), the potential performance benefits of KBs when provided as an exogenous fuel source has received little attention, but has been postulated (Cox & Clarke, 2014; Pinckaers *et al.* 2016). Apart from a role as an alternative fuel source, KBs may act as signalling molecules to regulate gene expression and adaptive responses (Shimazu *et al.* 2013; Zou *et al.* 2016). Moreover, therapeutic roles for KBs have long been proposed in a variety of disease states including aberrant glucose metabolism, genetic myopathies, hypoxic states and neurodegenerative pathologies (Veech, 2004). For therapeutic effects, exogenous ketones are ingested in the form of βHB salts or ketone esters to produce acute (~0.5 to 6 h) nutritional ketosis (Clarke *et al.* 2012; Kesl *et al.* 2016), but a surge in interest in KBs as a performance aid for athletes arose when ketone ester supplementation was confirmed in professional cycling (Abraham, 2015; Pinckaers *et al.* 2016). Moreover, a recent report provides the first evidence for acute nutritional ketosis achieved by ketone ester ingestion to alter the metabolic response to exercise and enhance exercise performance (Cox *et al.* 2016). Aspects of ketogenic diets, ketogenesis and ketone body metabolism have been reviewed elsewhere (Robinson & Williamson, 1980; Laffel, 1999; Paoli *et al.* 2013), so the present review will focus on the physiology of ketone bodies during and after exercise and in response to training, with specific interest in exploring the physiological basis for exogenous supplementation and potential benefits for performance and recovery in athletes.

Overview of ketone body metabolism

Ketone bodies in circulation. Plasma [KB] reflects the balance between hepatic production ('ketogenesis') and peripheral breakdown and utilisation ('ketolysis') in extra-hepatic tissues, both of which are under various levels of control as detailed in previous reviews (Robinson & Williamson, 1980; Laffel, 1999). Ketogenesis is an evolutionarily conserved adaptive response playing a critical role in survival during an energy crisis by providing a substrate for brain, which cannot utilise FFAs as a fuel source. AcAc, acetone, and βHB comprise the KBs, although βHB is not technically a ketone because the ketone moiety has been reduced to a hydroxyl group. AcAc and βHB are short-chain, four carbon organic acids that act as FFA-derived circulating substrates to provide energy to extra-hepatic tissues, whereas the contribution of acetone, readily generated by the spontaneous decarboxylation of AcAc, to energy provision is negligible. Plasma [KB] is <0.1 mM in the post-prandial state, whereas hyperketonaemia is accepted as [KB] exceeding 0.2 mM (Robinson & Williamson, 1980). Various states of CHO restriction, depletion and dysregulation produce hyperketonaemia to different degrees (Fig. 1).

Ketogenesis. The primary substrate for ketogenesis is FFAs liberated from adipose tissue. Ketogenic amino acids, namely leucine, lysine, phenylalanine, isoleucine, tryptophan, and tyrosine also serve ketogenesis, but are likely contribute to less than 5% of circulating KBs (Thomas *et al.* 1982). The rise in FFAs is consequent to the stimulation of lipolysis as a result of declines in plasma glucose and insulin that are characteristic of reduced CHO availability. Factors stimulating ketogenesis include an elevated glucagon-to-insulin ratio and decline in hepatic glycogen concentration, while reduced blood flow to the liver or elevations in [KBs] suppress ketogenesis (Robinson & Williamson, 1980; Laffel, 1999). Ketogenesis involves a series of sequential reactions beginning with acetyl CoA (Ac-CoA) and acetoacetyl CoA (AcAc-CoA), and ending with the liberation of AcAc (Fig. 2). Some AcAc is exported, but the majority is reduced to βHB in an NAD^+–NADH-coupled near equilibrium reaction catalysed by 3-hydroxybutyrate dehydrogenase (BDH), in which the equilibrium constant favours βHB formation. These KBs are transported into the circulation via the solute ligand carrier (SLC) protein 16A (SLC16A) family of monocarboxylate transporters (MCTs) in mitochondrial and sarcolemmal membranes.

Ketolysis in extra-hepatic tissues. In peripheral tissues, KBs, primarily in the form of βHB, enter the mitochondrial matrix again via MCT1-mediated

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transport. βHB is re-oxidised to AcAc via BDH after which sequential reactions result in the generation of two molecules of Ac-CoA (Fig. 2). These are incorporated into the TCA cycle via citrate synthase for terminal oxidation and production of ATP, which in skeletal muscle contributes to fuelling muscular work (Fery & Balasse, 1986, 1988). Succinyl-CoA:3-oxoacid CoA transferase (OXCT) is essential for ketolysis in extra-hepatic tissues, with very low abundance in hepatocytes explaining the lack of ketolytic activity in these cells (Robinson & Williamson, 1980).

Activity of OXCT is highest in heart and kidney, followed by skeletal muscle and the brain (Robinson & Williamson, 1980), but because skeletal muscle accounts for ~40% of body mass in adult humans, this organ accounts for the highest fraction of total KB metabolism at rest (Balasse & Fery, 1989; Laffel, 1999). Beginning almost 50 years ago, models using various durations of fasting, and combined with primed constant infusion of radio-labelled either AcAc or βHB tracers and arteriovenous difference measures to quantify KB turnover, established that skeletal muscle is a major site of ketolysis at rest (Hagenfeldt & Wahren, 1968; Owen & Reichard, 1971; Wahren *et al.* 1984; Elia *et al.* 1990; Mikkelsen *et al.* 2015). Skeletal muscle has a high affinity to KBs, but because of low circulating concentrations under normal conditions, the contribution to energy provision in muscle is less than 5%, and FFAs are the main source of energy provision in the post-absorptive state. The relationship between ketone oxidation and [KB] is curvilinear such that contribution to energy provision in skeletal muscle rises to ~10% after an overnight fast (Hagenfeldt & Wahren, 1968; Owen & Reichard, 1971), 20% to 50% after 72 h of fasting (Owen & Reichard, 1971; Elia *et al.* 1990), but declines to ~15% after 24 days of starvation (Owen & Reichard, 1971). Thus, skeletal muscle demonstrates saturation kinetics for the KB concentration–oxidation relationship, with saturation likely between 1 and 2 mM as demonstrated by fasting of various durations (compiled in Balasse & Fery, 1989) or step-wise βHB infusion (Mikkelsen *et al.* 2015).

Effect of aerobic exercise training on enzymes of ketogenesis and ketolysis

Adaptations to exercise training reduce perturbations to homeostasis during subsequent bouts of exercise, and thereby enhance resistance to fatigue. Central to these effects are enhanced respiratory capacity and contractile parameters, and importantly adaptations that contribute towards maximising delivery and utilisation of circulating substrates (reviewed in Egan & Zierath, 2013). Therefore, if KBs make a meaningful contribution to energy provision during exercise, it is pertinent to explore analogous regulation in skeletal muscle. Training-induced changes in expression and activities of enzymes of ketolysis in skeletal muscle have not been described in humans, but differences in KB metabolism during and after exercise between trained and untrained individuals have been reported (Johnson *et al.* 1969; Johnson & Walton, 1972; Rennie *et al.* 1974; Rennie & Johnson, 1974*a*). The general pattern is for attenuation in trained individuals of the post-exercise rise in [KB], but this is influenced by nutritional manipulation and relative exercise intensity, the latter of which has often been poorly controlled (see later sections).

Nevertheless, circulating concentrations reflect the balance between ketogenesis and ketolysis, these differences may be explained by the factors influencing one or both. For ketogenesis, data are limited but suggest that in exercise-trained rodents enzymatic activity of BDH or ACAT (Winder *et al.* 1974), or HMGCS (Askew *et al.* 1975) is unaltered in liver, and, in fact, the overall activity of the ketogenic pathway may be lower (El

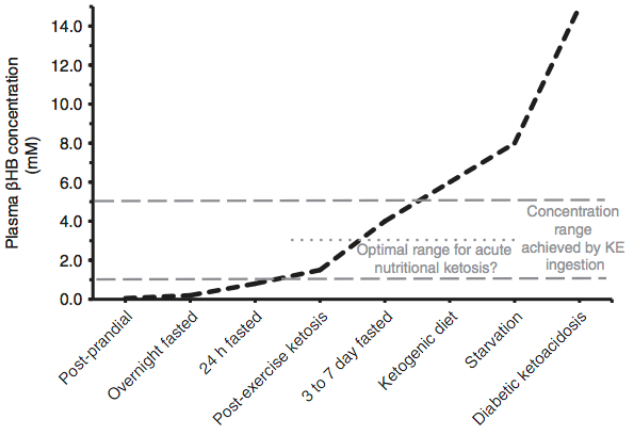

Figure 1. Changes in [βHB] under various physiological states
Plasma [KB] is <0.1 mM in the postprandial state when consuming high CHO or high protein meals, and rises upward after an overnight fast and with ketogenic dieting, prolonged fasting, starvation, and pathological states of ketoacidosis. After prolonged aerobic exercise, post-exercise ketosis (0.3 to 2.0 mM) may ensue depending on intensity and duration of exercise, aerobic fitness and nutrition status. The circulating KB ratio of βHB:AcAc is generally ~1:1 to 3:1, but during the aforementioned nutritional states can rise six- to tenfold, such that [KB] primarily reflects changes in [βHB]. An optimal concentration range for βHB to improve performance after exogenous ketone ingestion is proposed as ~1 to 3 mM, with concentrations ranging from ~1 to 5 mM reported after ketone ester (KE) ingestion. See text for further details.

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Midaoui *et al.* 2006) compared to untrained rodents. In these rodent models of intense aerobic exercise training, the activities of the ketolytic enzymes BDH, OXCT and ACAT are higher in trained skeletal muscle (Winder *et al.* 1974, 1975; Askew *et al.* 1975; Beattie & Winder, 1984). This coincides with two- to threefold higher *ex vivo* rates of βHB and AcAc oxidation in gastrocnemius muscle homogenates presented with concentrations of both βHB and AcAc at 0.1 and 0.5 mM (Winder *et al.* 1973, 1975).

In terms of muscle fibre type, enzymatic activities of BDH, OXCT and ACAT are all highest in type I fibres, intermediate in type IIA fibres, and lowest in type IIB fibres of rats (Winder *et al.* 1974). BDH is essentially undetectable in type IIB muscle fibres, and across the fibre types BDH activity is much lower than activities of OXCT and ACAT (Winder *et al.* 1974). Although OXCT is essential for ketolysis, BDH activity is, therefore, potentially rate limiting in skeletal muscle. When rats performed 12 weeks of treadmill running, compared to sedentary rats BDH activity was almost threefold higher in type I fibres, but sixfold higher in type IIA fibres of trained skeletal muscle, resulting in levels comparable to the type I fibres (Winder *et al.* 1974). OXCT activity was 26% higher in type I, and approximately twofold higher in type IIA and IIB fibres, whereas ACAT activity was 40% to 45% higher in all three fibre types in trained skeletal muscle (Winder *et al.* 1974). Similarly, in skeletal muscle from mice with 8 weeks of access to running wheels, the difference compared to sedentary mice was greater for BDH mRNA expression

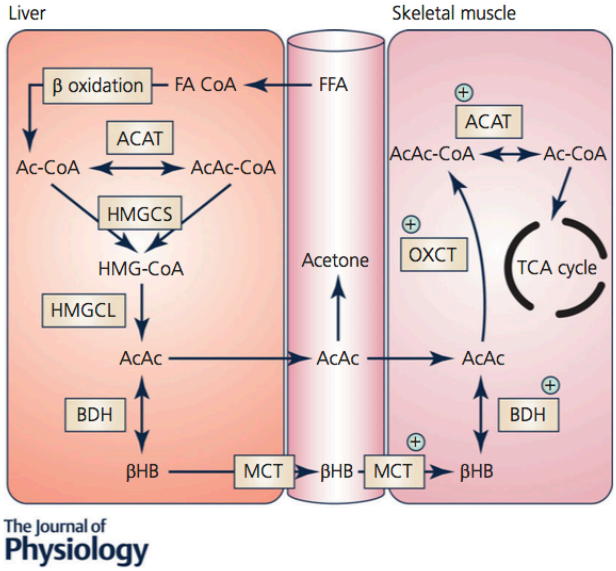

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Figure 2. Metabolic pathways of ketone body metabolism in liver and skeletal muscle
Ketogenesis: FFAs are converted to fatty acyl CoA (FA-CoA), enter hepatic mitochondria via CPT1-mediated transport and undergo β-oxidation to acetyl CoA. Sequential reactions of condensation of Ac-CoA molecules to acetoacetyl CoA (AcAc-CoA) by mitochondrial thiolase activity of Ac-CoA acetyltransferase (ACAT), generation of hydroxymethylglutaryl-CoA (HMG-CoA) by hydroxymethylglutaryl CoA synthase (HMGCS), and decomposition of HMG-CoA, liberating AcAc and Ac-CoA, in a reaction catalysed by HMG-CoA lyase (HMGCL). AcAc is the central KB, and some will be exported to the circulation but the majority is reduced to βHB in an NAD^+–NADH-coupled near equilibrium reaction catalysed by BDH, in which the equilibrium constant favours βHB formation. Ketolysis: The only metabolic fate of βHB is inter-conversion with AcAc, and upon entry into peripheral tissues it is re-oxidised to AcAc. Covalent activation of AcAc by CoA is catalysed by succinyl-CoA:3-oxoacid CoA transferase (OXCT) resulting in generation of AcAc-CoA. This near equilibrium reaction exchanges CoA between succinate and AcAc, with succinyl-CoA acting as a CoA donor. Because the free energy released by hydrolysis of AcAc-CoA is greater than that of succinyl-CoA, the equilibrium of this reaction thermodynamically favours the formation of AcAc. Two molecules of Ac-CoA are liberated by thiolytic cleavage of AcAc-CoA by ACAT, after which Ac-CoA is incorporated into the TCA cycle. Protein content and enzyme activity that are higher in exercise-trained skeletal muscle are indicated by the green cross (+).

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(~twofold higher than sedentary) compared to differences in OXCT and ACAT mRNA expression (~30% to 50% higher) (Svensson *et al.* 2016). These changes in ketolytic enzymes are localised to the working muscle given the absence of change after training in the heart (Askew *et al.* 1975), kidney and brain (Winder *et al.* 1974).

In terms of KB transport into skeletal muscle, similarly to the ketolytic enzymes, MCT1 protein expression is highest in type I fibres, poorly expressed in type II fibres, and correlates well with muscle oxidative capacity (Bonen, 2001). Elevated MCT1 protein expression after exercise training is well-established for human skeletal muscle, and increases occur in an intensity-dependent manner (Thomas *et al.* 2012). Using a rodent perfused hindlimb model, the capacity for uptake of KBs in skeletal muscle at 1 mM each of βHB and AcAc was higher in an aerobically trained group of rats, with uptake of total KB, AcAc and βHB 33%, 27% and 53% higher, respectively, compared to untrained rats (Ohmori *et al.* 1990). Similarly, βHB clearance during a βHB tolerance test is higher in mice given 8 weeks of running wheel access, or with enhanced oxidative capacity consequent to skeletal muscle over-expression of PGC-1α, a transcriptional co-activator and master regulator of mitochondrial biogenesis in adaptive responses such as exercise training (Svensson *et al.* 2016). In both conditions, this coincides with elevated expression of MCT1 and the ketolytic enzymes in skeletal muscle. Therefore, the uptake and utilisation of KBs in skeletal muscle is likely to be greatest in those individuals that are highly trained with a high proportion of type I muscle fibres and a high oxidative capacity in skeletal muscle.

Ketone body metabolism during exercise

The existing literature on fuel selection during exercise has focused almost exclusively on utilisation of CHO and fat, but skeletal muscle has the ability to resynthesize ATP from other substrates including protein, lactate and KBs (Fery & Balasse, 1986, 1988; Mazzeo *et al.* 1986; Wagenmakers *et al.* 1991). With increasing exercise intensity, the contribution of substrates to energy provisions shifts from blood-borne FFAs and glucose to increased reliance on intramuscular fuel stores, namely intramuscular triglyceride (IMTG) and muscle glycogen, such that at moderate to high intensities (>75% $\dot{V}_{O_2max}$) of exercise, muscle glycogen is the main source of energy provision (van Loon *et al.* 2001). This pattern is readily altered by nutritional manipulation such as CHO loading and acute CHO ingestion resulting in increased CHO utilisation (Bosch *et al.* 1996), glycogen depletion resulting in increased contribution of protein to energy provision (Wagenmakers *et al.* 1991), and habitual high fat consumption resulting in increased contribution of fat to energy provision (Volek *et al.* 2016). Clearly, skeletal muscle is a major site of ketolysis under fasting conditions, but central to the rationale for exogenous ketone supplementation must be the observations that ketolysis increases during exercise, makes a meaningful contribution to energy provision, and can alter patterns of substrate utilisation.

The pioneering work of Hagenfeldt, Wahren and colleagues (Hagenfeldt & Wahren, 1968, 1971; Wahren *et al.* 1984) and Fery, Balasse and colleagues (Balasse *et al.* 1978; Fery & Balasse, 1983, 1986, 1988) established that KB disposal into human skeletal muscle is elevated as much as fivefold during exercise. This is generally reflected by a drop in [KB] soon after the onset of exercise, primarily βHB, concomitant with increases in KB oxidation in skeletal muscle and elevated metabolic clearance rate (MCR). MCR is a measure of the ability of tissues to remove ketones from the blood, analogous to arteriovenous difference per unit time, but when measured during exercise is taken to represent an index of the ability of exercise to stimulate the capacity of working muscles to extract and utilise ketones (Fery & Balasse, 1983; Balasse & Fery, 1989). Because the stoichiometry of KB oxidation yields respiratory quotients of 1.00 and 0.89 for AcAc and βHB, respectively (Frayn, 1983), calculation of oxidation rates for KBs from whole-body gas exchange data has not been routinely performed using methods that determine the relative contribution of CHO and fat oxidation. However, a recent attempt has been made (Cox *et al.* 2016) based on methods and assumptions described for KB utilisation during ketogenesis (Frayn, 1983). Previous to this, oxidation rates for KBs have historically been derived from arteriovenous differences of radiolabelled KBs across working muscles with rates calculated as a fraction of O_2 consumption or CO_2 production (Hagenfeldt & Wahren, 1968; Balasse *et al.* 1978).

Like CHO and fat utilisation, KB metabolism during exercise is influenced by a variety of factors including metabolic status (Wahren *et al.* 1984; Fery & Balasse, 1986), training status (Johnson & Walton, 1972; Rennie *et al.* 1974; Beattie & Winder, 1985), and the intensity of exercise (Cox *et al.* 2016). Given the aforementioned fibre type-specific differences for activities of ketolytic enzymes, the muscle fibre type profile of the working muscle is also likely to be an important determinant of ketolysis during exercise. However, the most important determinant of KB metabolism during exercise is the degree of ketonaemia, and the method by which this is achieved, i.e. of endogenous or exogenous origin.

Ketone body metabolism during exercise under conditions of endogenous ketosis. Like KB metabolism in resting skeletal muscle, the relationship between concentration and oxidation or MCR is curvilinear (reviewed in Balasse & Fery, 1989). At low ketonaemia (<1.0 mM) such as that produced by an overnight fast, resting MCR is as much as fourfold greater than

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during prolonged fasting (Fery & Balasse, 1983). During prolonged exercise of low-to-moderate intensity after an overnight fast, MCR increases by 50% to 75% (Fery & Balasse, 1983, 1986), which indicates that working muscle has an increased capacity to extract ketones from blood compared to rest. However, when ketonaemia exceeds 2.5 mM such as that achieved by greater than 72 h of fasting, the exercise-induced rise in MCR is abolished (Fery & Balasse, 1986). Therefore, when ketosis is achieved by prolonged (>72 h) fasting there is a negligible contribution of KB oxidation to energy provision (Hagenfeldt & Wahren, 1971; Fery & Balasse, 1986), but after an overnight fast, the contribution ranges from 2 to 10% (Balasse *et al.* 1978; Fery & Balasse, 1983; Wahren *et al.* 1984). Under these conditions, the majority of energy provision in working muscle is from CHO and fat as classically described (van Loon *et al.* 2001). Moreover, unlike CHO and fat, there is progressive attenuation of the oxidation of KBs with rising ketonaemia, and thus the mobilisation of KBs is not the factor limiting oxidation in skeletal muscle. This attenuation of exercise-stimulated MCR suggests either that above a threshold concentration the capacity for skeletal muscle to oxidise KBs becomes saturated, and/or that hyperketonaemia itself is a self-inhibitory factor (Balasse & Fery, 1989). Mechanistically, this is likely to be mediated either through the inhibition of OXCT by elevated AcAc, and/or via FFA-mediated inhibition of ketolysis (Robinson & Williamson, 1980). This regulation is critical in the starvation response because the capacity of the liver to produce KBs closely matches the requirements of the brain to utilise KBs as an energy source (Robinson & Williamson, 1980). Therefore, excessive oxidation by working muscle would threaten survival, whereas its inhibition spares circulating substrate for the brain (Hagenfeldt & Wahren, 1971; Fery & Balasse, 1983).

Methods of exogenous ketone supplementation producing acute nutritional ketosis

Investigating effects of ketosis on skeletal muscle metabolism has been typically achieved by endogenous ketosis using fasting of various durations (Balasse & Fery, 1989), or by exogenous ketosis produced by either ketone salt ingestion (Johnson & Walton, 1972), or infusion of AcAc or βHB (Fery & Balasse, 1988; Mikkelsen *et al.* 2015). Endogenous ketosis may also be achieved by CHO restriction, particularly by ketogenic diets (Paoli *et al.* 2013). The practical relevance for athletes seeking performance gains of metabolic responses generated from prolonged fasting is negligible, whereas benefits of ketogenic dieting for performance with a high intensity component are equivocal (Burke, 2015). This has led to the exploration of exogenous ketone ingestion as a means to achieve acute nutritional ketosis. Importantly, because endogenous ketosis results in concomitant elevations in FFAs and alterations in glucose, insulin and counter-regulatory hormones, isolating the metabolic effects specific to KBs has proved challenging. Therefore, exogenous ketone supplementation is a means to address these questions and explore potential for performance and therapeutic benefits.

Oral administration of KBs in their free acid form is expensive and ineffective at producing ketosis, so buffering the free acid form with sodium/potassium/calcium salts has been explored and these compounds are commercially available. These too are relatively ineffective at increasing [βHB], but may be improved by co-ingestion with medium chain triglycerides (C:8, C:10), at least in rats (Kesl *et al.* 2016). However, ingestion of large quantities of KB salts is impractical due to resulting gastrointestinal distress, and potentially undesirable consequences of cation overload or acidosis (Veech, 2004).

The development of ketone esters provides an alternative method to increase [βHB], which is well-tolerated in rodents and humans (Clarke *et al.* 2012; Cox *et al.* 2016; Kesl *et al.* 2016). Two prominent ketone esters in the published literature are the *R,S*-1,3-butanediol acetoacetate diester (Kesl *et al.* 2016) and the *(R)*-3-hydroxybutyl *(R)*-3-hydroxybutyrate ketone monoester (Clarke *et al.* 2012; Cox *et al.* 2016). Acute ingestion of either ester can result in short-term (~0.5 to 6 h) nutritional ketosis indicated by [βHB] >1 mM (Clarke *et al.* 2012; Kesl *et al.* 2016). For the ketone monoester, ingestion at a dose of 573 mg (kg body mass (BM))$^{-1}$ resulted in [βHB] of ~3 mM after 10 min and rising to ~6 mM 30 min after ingestion (Cox *et al.* 2016). Nutritional ketosis is therefore achieved without the impracticality of prolonged fasting or ketogenic dieting.

Ketone body metabolism during exercise under conditions of exogenous ketosis. The aforementioned self-inhibitory effect of rising ketonaemia underscores a key methodological issue when considering KB metabolism in skeletal muscle, namely the method of achieving ketosis. While fasting of various durations is a widely used model of ketosis, acute nutritional ketosis relevant to sports performance would be achieved with replete glycogen stores, and in the absence of prolonged elevations in FFAs and [KB] that would be likely to impair KB oxidation rates through these mechanisms. To our knowledge, only two studies have addressed this convincingly by examining effects of exercise on KB metabolism without interference from the various hormonal and metabolic perturbations associated with prolonged fasting or diabetes (Fery & Balasse, 1988; Cox *et al.* 2016).

In the former study (Fery & Balasse, 1988), infusion of sodium AcAc after an overnight fast achieved [KB]

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of ~6 mM (βHB ~3.5 mM, AcAc ~2.5 mM) at the onset of 2 h of exercise at ~52% $\dot{V}_{O_2max}$. Notably, AcAc did not change during exercise whereas βHB declined throughout exercise, to be reduced by ~2 mM at the end of exercise. This coincided with a progressive rise in MCR throughout exercise, peaking at ~75% higher than rest at the end of exercise. In contrast, this effect was abolished with similar ketonaemia in 3–5 day fasted participants. Importantly, although the inhibition of KB oxidation by hyperketonaemia is present during exogenous ketosis, an 'auto-amplification' was noted that is not present in fasting ketosis, i.e. the initial rise in MCR induced by exercise causes a reduction in concentration which, in turn, provokes a further rise in MCR and so on. Additionally, the threshold concentration at which hyperketonaemia inhibits MCR was higher in exogenous ketosis than in fasting ketosis. However, in terms of contribution to energy provision, this ultimately only resulted in a 2% contribution over the 2 h exercise bout. Nevertheless, plasma [lactate] did not rise during exercise after AcAc infusion compared to a ~1 mM rise in the fasted participants, which suggests that despite a modest contribution to energy provision, exogenous ketosis can impact on metabolic processes during exercise.

Despite this promise, these data remained largely isolated for almost 30 years with the exception of a couple of obscure reports that admittedly did recapitulate the effects of βHB to alter the metabolic response to very intense exercise in rats (Kamysheva & Ostrovskaia, 1980), and ischaemic exercise in humans (Lestan *et al.* 1994). The latter report, in fact, supported the ability of a modest elevation in βHB (~0.5 mM) via infusion of sodium βHB to reduce the plasma lactate response to exercise in an ischaemic forearm model. However, with the development of the *(R)*-3-hydroxybutyl *(R)*-3-hydroxybutyrate ketone monoester (KE), a comprehensive investigation of substrate metabolism in highly trained athletes in the presence of acute nutritional ketosis has recently been published (Cox *et al.* 2016).

In one of a series of experiments, ingestion of KE resulted in acute nutritional ketosis indicated by [βHB] of ~3 mM after 10 min and rising to ~6 mM 30 min after ingestion. During exercise lasting 45 min at either 40% or 75%W_{max}, [βHB] was ~2 and 3 mM, respectively, lower than ketosis produced after ingestion at rest. This provided the first evidence of intensity-dependent disposal of βHB during exercise. Moreover, based on expired air analysis adjusted for oxidation of KBs, βHB oxidation contributed 18% and 16% of oxygen consumption to energy provision at the respective intensities. The larger than previously reported contribution of βHB oxidation probably reflects the fact that the participants in these experiments were highly trained cyclists, therefore with a greater capacity of skeletal muscle to uptake and oxidise KBs. Moreover, this model is markedly different to the fasting-induced ketonaemia and the associated self-inhibitory regulation so making direct comparisons are difficult. In a separate exercise bout lasting 60 min at 75% $\dot{W}_{max}$ and with similar [βHB] after KE ingestion, the rise in plasma [lactate] was blunted by ~2 to 3 mM (~50% reduction) compared to ingestion of an isocaloric CHO drink. Subsequent experiments with ingestion of the KE demonstrated inhibition of glycolytic metabolism, sparing of muscle glycogen, reduced deamination of branched-chain amino acids, and increased reliance on IMTG during exercise (Cox *et al.* 2016). Lastly, after a 60 min pre-load at 75% $\dot{W}_{max}$, cycling performance in a 30 min time-trial was improved by 2% (411±162 m; mean ± SEM, $n = 8$) with KE + CHO compared to isocaloric CHO ingestion. The KE + CHO fuelling strategy combined KE (40%; 573 mg (kg BM)$^{-1}$) with CHO (60%) and elevated [βHB] to between ~1.5 and 3 mM throughout. Importantly, the KE + CHO condition provided CHO at a minimum rate of 1.2 g min^{-1}, consistent with an optimal CHO-based fuelling strategy (Burke, 2015). Taken together, these data suggest that acute nutritional ketosis by consumption of exogenous ketones has dramatic effects on skeletal muscle metabolism during exercise, and can confer a performance benefit to elite athletes (Fig. 3). The positive findings notwithstanding, potential adverse effects should be considered for any performance aid prior to adoption. Side-effects of KE ingestion have been reported in humans (Clarke *et al.* 2012). Specifically, in a repeated dose design over 5 days, adverse effects such as flatulence, nausea, diarrhoea and dizziness were reported in five out of twenty-four participants at doses ranging from 420 to 1071 mg (kg BM)$^{-1}$. Such issues were prevalent in almost all participants when the dose was increased to 2142 mg (kg BM)$^{-1}$ per day, indicating a possible upper limit of tolerability in adults (Clarke *et al.* 2012). Therefore, these data combined with the dosing strategy associated with exercise performance benefits should be used to guide future investigations on ergogenic potential.

Ketone body metabolism after exercise: post-exercise ketosis

Despite the aforementioned decline in [KB] at the onset of exercise, this pertains to situations where exercise has begun during hyperketonaemia (Balasse *et al.* 1978; Fery & Balasse, 1983, 1988; Cox *et al.* 2016). In the post-absorptive state, the pattern generally observed is for [KB] to rise gradually during prolonged exercise up to 0.2 to 0.4 mM, after which time post-exercise ketosis (PEK) of 0.3 to 2.0 mM is observed for several hours into recovery (Koeslag, 1982). Explained in terms of plasma kinetics, at cessation of exercise, the rate of appearance of KBs increases coincident with a decrease in MCR relative to rates present during exercise. MCR remains above resting

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values for several hours after exercise, but ketogenesis exceeds ketolysis during this period.

On a mechanistic level, regulation probably resides at several sites including malonyl CoA-mediated regulation of fat transport into hepatocytes via CPT-1 in addition to availability of Ac-CoA for ketogenesis, and oxaloacetate for the TCA cycle as classically described for ketogenic regulation. Because oxaloacetate is a product of pyruvate formed during glycolysis, reductions in glycolytic flux with low glycogen content after intense exercise result in oxaloacetate moving to cytoplasm for preferential use in gluconeogenesis, which allows diversion of Ac-CoA towards ketogenesis during the post-exercise period rather than to citrate synthesis for the TCA cycle. Additionally, the actions of insulin and glucagon exert a strong influence through activation and inhibition, respectively, of Ac-CoA carboxylase (ACC), which catalyses the synthesis of malonyl CoA from Ac-CoA. When liver glycogen becomes depleted and glucagon:insulin ratio is elevated, the synthesis of malonyl CoA is reduced, thereby relieving the inhibition of fat transport into hepatocytes, and resulting in elevated levels of Ac-CoA. These regulatory mechanisms are acutely sensitive to nutrient manipulations before and after exercise and to aerobic exercise training, given their respective influences on substrate availability and utilisation during exercise.

Modulation of post-exercise ketosis by aerobic exercise training and nutrition intervention. An attenuation of, or abolished, post-exercise ketosis has been consistently observed in rodents and humans in response to aerobic exercise in trained *versus* untrained individuals (Johnson *et al.* 1969; Johnson & Walton, 1972; Rennie *et al.* 1974), or after a period of exercise training (Rennie & Johnson, 1974*a*; Beattie & Winder, 1984, 1985; Adams & Koeslag, 1988, 1989; Ohmori *et al.* 1990). The aforementioned enhanced ketolytic capacity and down-regulation of ketogenic capacity by training may play a role in these observations, but the majority of this work has been performed in comparisons, with the absolute exercise intensity and duration being the same for comparisons (reviewed in Koeslag, 1982). This is problematic because the relative exercise intensity is the key determinant of the metabolic and hormonal response to acute exercise, e.g. catecholamine responses, FFA mobilisation and glycogen utilisation among others. When trained and untrained participants have performed exercise at a similar relative intensity, PEK is blunted but not abolished in trained individuals (Rennie *et al.* 1974). Moreover, in rodents when exercise is completed to exhaustion, i.e. the trained rats exercise for longer than untrained, [βHB] is ~twofold higher at the exercise cessation in the trained group (Askew *et al.* 1975). These divergent findings are likely to be due to the degree of liver glycogen depletion that occurs (Adams & Koeslag, 1988), inasmuch as higher levels of resting liver glycogen and attenuated rates of depletion are a consequence of training (Baldwin *et al.* 1975).

Therefore, PEK is strongly influenced by nutrition manipulation. High CHO feeding prior to exercise attenuates PEK regardless of training status (Rennie &

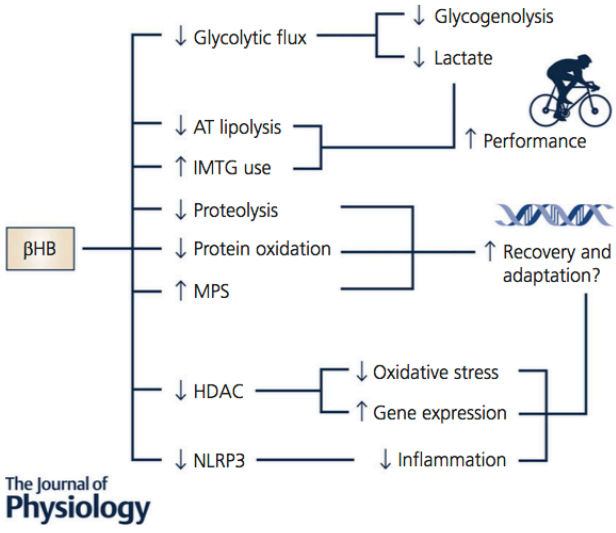

Figure 3. βHB as a metabolic regulator and signalling metabolite
Effects of elevating βHB through acute nutritional ketosis may be mediated by acute regulation of substrate utilisation that may enhance performance, and/or possibly through regulation of recovery and adaptive processes related to inflammation, oxidative stress and changes in gene expression. See text for further discussion. AT, adipose tissue; HDAC, histone deacetylase; IMTG, intramuscular triglyceride; MPS, muscle protein synthesis.

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Johnson, 1974*b*; Askew *et al.* 1975; Koeslag *et al.* 1980), and CHO restriction increases PEK (Impey *et al.* 2016). Glucose ingestion at 2 h into recovery (Koeslag *et al.* 1982; Carlin *et al.* 1987) and alanine during recovery (Koeslag *et al.* 1980, 1985; Carlin *et al.* 1987) attenuate PEK, but the glucose effect is not seen when glucose is ingested immediately after exercise. Alanine ingestion increases mitochondrial [oxaloacetate] in liver, thereby allowing condensation with Ac-CoA and diversion away from ketogenesis. This suggests that the early PEK response is determined by the extent of liver glycogen depletion and reduced glycolytic flux, whereas several hours into recovery it is under regulation by insulin and [FFA] related to nutrition intake.

Metabolic consequences of post-exercise ketosis during recovery: a role for exogenous ketones as a recovery aid? The physiological role for PEK is likely to favour the replenishment of muscle glycogen, consistent with classically described metabolic actions of ketosis in the sparing of protein and CHO stores during times of low CHO availability. During the post-exercise recovery period, in contrast to the reliance on CHO metabolism during exercise, muscle glycogen resynthesis has a high metabolic priority and is facilitated by an increase in fat oxidation and sparing of CHO sources for energy provision (Kiens & Richter, 1998). A priority for muscle glycogen resynthesis over liver glycogen resynthesis is suggested to occur because in ancestral terms, a depleted liver is less of a hindrance to intense exertion than depleted muscle (Adams & Koeslag, 1988). To this end, the priority for muscle glycogen resynthesis is observed even during CHO restriction (Adams & Koeslag, 1989), and is achieved through non-CHO sources such as lactate and alanine being used for hepatic gluconeogenesis and redistribution to skeletal muscle (Fournier *et al.* 2002). The contribution of PEK may be via the ability of KBs to inhibit glycolysis and increase the conversion of glucose to glycogen as demonstrated in rat skeletal muscle *in vitro* (Maizels *et al.* 1977), and a perfused heart model in dogs (Laughlin *et al.* 1994). This effect is likely to be mediated by inhibition of PDH and phosphofructokinase (PFK) by elevations in Ac-CoA and citrate formation, respectively, as a consequence of metabolism of AcAc in mitochondria (Randle *et al.* 1964; Maizels *et al.* 1977; Laughlin *et al.* 1994; Kashiwaya *et al.* 1997).

This raises the possibility that an optimal post-exercise recovery milieu exists that includes both CHO and ketones to enhance recovery of muscle glycogen. This is not possible by conventional nutrition strategies because elevations in glucose, lactate and alanine ultimately limit ketogenesis and PEK. The suggestion is that the co-ingestion of exogenous ketones and CHO in a recovery protocol can confer a metabolic advantage. This hypothesis remains to be tested rigorously, but a preliminary report describes a 33% increase in glucose disposal and 50% increase in muscle glycogen content after 2 h of recovery when nutritional ketosis (~5 mM βHB) is superimposed on a hyperglycaemic (10 mM glucose) clamp in well-trained military servicemen (Holdsworth *et al.* 2016).

Repletion of muscle glycogen is only one component of post-exercise recovery, and nutrition strategies for recovery include protein ingestion, with the aim to limit muscle protein breakdown and enhance muscle protein synthesis (MPS). KBs have protein sparing effects in skeletal muscle as indicated by reduced alanine release during starvation (Sherwin *et al.* 1975), and reduced leucine oxidation (Nair *et al.* 1988). In the latter study, this coincided with a 10% increase in MPS measured by fractional synthesis rate and occurred with [βHB] of ~2 mM achieved via sodium βHB infusion. This raises the possibility that acute nutritional ketosis can complement current strategies for optimising MPS in the post-exercise period. Additionally, because low CHO stores during exercise lead to elevated rates of protein oxidation (Wagenmakers *et al.* 1991), exogenous ketone supplementation may provide both a fuel source and contribute to protein sparing and recovery during training in CHO-restricted states commonly practiced by athletes (reviewed in Bartlett *et al.* 2015). Together with the preliminary data for muscle glycogen resynthesis, this suggests that post-exercise recovery is another application where elite athletes may benefit from exogenous ketone supplementation, and where future research is warranted.

Effects beyond fuelling: βHB as a HDAC inhibitor

As investigative techniques in molecular biology evolve, so too does our appreciation of how complex integrative signalling networks regulate skeletal muscle adaptation in response to stimuli such as nutrient manipulation and exercise training (Egan & Zierath, 2013). Previously considered relatively inert outside their primary metabolic function, numerous substrates and metabolites are emerging as important regulators of intracellular signalling and tissue adaptation (Hashimoto *et al.* 2007; Gao *et al.* 2009; Morton *et al.* 2009; Roberts *et al.* 2014). Noteworthy for the present review is the recent identification of AcAc as a regulator of skeletal muscle satellite cell proliferation and muscle regeneration (Zou *et al.* 2016), and βHB as an inhibitor of HDACs (Shimazu *et al.* 2013) and the NLRP3 inflammasome (Youm *et al.* 2015). The latter observations are a consequence of βHB, in essence, acting as a signalling metabolite to regulate gene expression and metabolic processes (Fig. 3).

Histone acetyltransferases (HATs) and HDACs are enzymes that facilitate the addition or removal, respectively, of acetyl moieties from specific lysine residues on histones and target proteins (McKinsey

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et al. 2001). In general, hyperacetylation of histone tails induces transcriptional activation while hypoacetylation is associated with transcriptional repression. Class IIa HDACs (HDAC4, -5, -7 and -9) are highly expressed in skeletal muscle (McKinsey *et al.* 2001) and their function is responsive to both aerobic endurance exercise in humans (McGee *et al.* 2009; Egan *et al.* 2010) and nutritional intervention in rodents (Gao *et al.* 2009; Shimazu *et al.* 2013). An acute bout of aerobic exercise increases class IIa HDAC phosphorylation and subsequent nuclear exclusion, thus inhibiting HDAC-mediated repression of specific exercise-responsive genes such as GLUT4 and PGC-1α (McGee & Hargreaves, 2004; McGee *et al.* 2009; Egan *et al.* 2010). This suggests that compounds that inhibit or disrupt HDAC inhibition could be used to mimic or enhance adaptations to exercise.

Regulation of HDAC activity by nutrients including butyrate and βHB has also been established (Gao *et al.* 2009; Shimazu *et al.* 2013). Butyrate, a short chain fatty acid formed via the fermentation of indigestible dietary fibres by microbial species in the gut, is a potent inhibitor of HDAC activity (Gao *et al.* 2009). Mice supplemented with sodium butyrate are resistant to diet-induced obesity, and have elevations in markers of skeletal muscle mitochondrial biogenesis analogous to exercise effects (Gao *et al.* 2009). βHB is structurally similar to butyrate, and although not as potent as butyrate, also inhibits HDAC class I and II activity in a dose-dependent manner and supressed oxidative stress responses (Shimazu *et al.* 2013). Importantly, HDAC inhibition by βHB both *in vitro* and *in vivo* is evident at physiologically relevant concentrations of βHB, i.e. 1 to 4 mM, which is similar to those attained during fasting, PEK and exogenous ketone ingestion (Fig. 1; Clarke *et al.* 2012; Kesl *et al.* 2016). However, although the inhibitory effects were observed in multiple tissues, they remain to be confirmed in skeletal muscle. If confirmed, it will be intriguing to explore whether, apart from the aforementioned ergogenic effects, exogenous ketone supplementation complements exercise-mediated adaptive changes associated with modulating HDAC function (Fig. 3).

Exogenous ketone supplementation for athletes: cautionary notes and future directions

Despite a strong physiological basis for a variety of benefits for performance and recovery, the relatively recent availability of exogenous ketones and thus far only one peer-reviewed paper examining exercise metabolism, performance and nutritional ketosis, means that much more research remains to be performed (Pinckaers *et al.* 2016). The central tenet is that the combination of fuel sparing and improved energetic efficiency during acute nutritional ketosis confers performance benefits (Fig. 3). Alterations in fuel selection during steady-state exercise have been demonstrated, which indicate reduced glycolytic flux, sparing of CHO and increased contribution of IMTG and βHB to energy provision (Cox *et al.* 2016). Whether this sparing of CHO, in fact, manifests as *impaired* CHO utilisation remains to be determined. The mechanistic basis for CHO sparing by exogenous ketones is presently proposed as inhibition of glycolytic flux via inhibition of PDH and PFK by increases in NADH:NAD^+, acetyl-CoA:CoA ratio or citrate. In theory, this could be problematic for sports that rely heavily on contributions from glycolytic pathways, or a range of sports that are intermittent and/or require periods of high intensity 'bursts' on a moderate intensity background. This is analogous to the lack of performance benefits for most athletes undertaking low CHO, high fat diets (Burke, 2015). In fact, impaired performance during high intensity efforts has been observed under such conditions (Havemann *et al.* 2006), and may be explained by sustained attenuation of PDH activity (Stellingwerff *et al.* 2006). Whether the same effects are observed with acute nutritional ketosis given that this is a very different metabolic milieu, especially in the context of exercise, remains to be explored.

The metabolic consequences of inhibition of adipose tissue lipolysis by KBs also warrants further exploration, given that this process is an important contributor to circulating FFAs, and therefore to the contribution of fat oxidation to energy provision during long duration, submaximal exercise. Nutritional ketosis achieved by either AcAc infusion (Fery & Balasse, 1988) or KE ingestion (Cox *et al.* 2016) inhibits the lipolytic effect of exercise, i.e. the amount of lipid-derived substrates available for working muscle is reduced. In the latter study, this did not manifest as increased glycogenolysis and/or glucose utilization, despite these usually being accelerated by the inhibition of FFA availability (van Loon *et al.* 2005). In fact, glycogenolysis was attenuated and IMTG utilisation was increased in the KE experiments (Cox *et al.* 2016), suggesting differential regulation to that achieved by nicotinic acid administration (van Loon *et al.* 2005). However, in each of the experimental conditions with KE, the duration of exercise was between 45 and 120 min at moderate intensity (Cox *et al.* 2016). Recently, the inhibition of lipolysis via nicotinic acid impaired cycling time-trial performance in long (120 min), but not shorter (60 and 90 min) duration efforts (Torrens *et al.* 2016). Thus, even in events with high CHO dependence (~80 to 95% of energy provision), inhibition of lipolysis may impair endurance performance, particularly in long duration activities analogous to professional cycling or triathlon. Clearly, the many nodes of metabolic regulation influencing skeletal muscle fuel selection that are altered by nutritional ketosis need to be fully elucidated before sports-specific ergogenic strategies can be advised.

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Improved energetic efficiency is an often-cited potential benefit of acute nutritional ketosis (Veech, 2004; Cox & Clarke, 2014). In this model, exogenous ketones may provide thermodynamic advantages over CHO and fat, because the available free energy to perform work, the free energy of ATP hydrolysis ($\Delta G'_{ATP}$), is greater with KBs, and require less oxygen per mole of carbon to oxidise. Support for this hypothesis comes from a perfused working rat heart model where adding KBs to the perfusate supressed glycolytic flux, and increased hydraulic efficiency (expressed as work in J (mol O_2 consumed)$^{-1}$) by 28% (Sato *et al.* 1995; Kashiwaya *et al.* 1997). In practical terms, if the same effect occurs in skeletal muscle, this would translate as a higher power output for the same oxygen consumption (i.e. improved muscular efficiency) during exercise with nutritional ketosis, but this remains unexplored at present.

Because KBs serve as a substrate for the brain, and therapeutic uses for KBs have been proposed for cognitive enhancement and neurodegenerative pathologies (Veech, 2004), the central nervous system (CNS) may be another target for performance-enhancing effects of nutritional ketosis. Although speculative at present, effects related to motor recruitment, perceived exertion, pacing strategies, skill execution, reaction time, and decision-making will be interesting for future research, in addition to the proposed role for the CNS in regulating performance beyond effects related to skeletal muscle metabolism (Noakes, 2011).

As with any ergogenic aid or nutrition strategy, optimising dosing strategies including quantity and timing will be important. Given the saturation kinetics of KB oxidation by skeletal muscle and curvilinear relationship between oxidation and plasma concentrations, it is likely that there is an optimal range for performance benefits. At present, we speculate that this exists between 1 and 3 mM βHB. As with many ergogenic acids, more is unlikely to be better and may even be deleterious given the potential for acidosis at higher [KB], and aforementioned gastrointestinal distress and other side-effects sometimes observed with KE, so careful consideration should be given to these issues.

In conclusion, although data are preliminary, acute nutritional ketosis achieved by exogenous ketone supplementation has the potential to alter fuel selection during exercise and confer performance benefits. This is most likely to be the case in trained individuals who have a greater capacity to take up and oxidise KBs during exercise as a result of training. Additionally, a strong physiological basis exists that suggests potential benefits for supporting training and recovery. While much work remains to be performed, particularly in relation to sport-specific strategies, this promises to be an exciting topic for scientists, practitioners and athletes alike for the coming years.

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Additional information

Competing interests

The authors declare no conflict of interest.

Author contributions

B.E. conceived the review and drafted the outline. M.E., K.E.C. and B.E. drafted the initial manuscript, revised and finalised the content. All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Appendix B: Published European Journal of Sport Science article

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Effect of acute ingestion of β-hydroxybutyrate salts on the response to graded exercise in trained cyclists

Mark Evans, Ella Patchett, Rickard Nally, Rachel Kearns, Matthew Larney & Brendan Egan

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during periods of low glucose availability such as during fasting, starvation, and ketogenic diets (Balasse & Féry, 1989; Laffel, 1999; Robinson & Williamson, 1980). Although principally acting as an alternative fuel source for the brain when glucose concentrations are diminished, ketone bodies are also used by skeletal muscle to provide up to 10% of energy during exercise in the fasted state (Balasse, Fery, & Neef, 1978; Féry & Balasse, 1983; Fery, Franken, Neef, & Balasse, 1974; Wahren, Sato, Ostman, Hagenfeldt, & Felig, 1984). However, the direct contribution to energy provision may be secondary to the potential metabolic action of supplemental ketones. For instance, ketone bodies have wide-ranging metabolic effects on peripheral tissues such as glucose sparing, anti-lipolytic effects, and stimulation of muscle protein synthesis (Maizels, Ruderman, Goodman, & Lau, 1977; Mikkelsen, Seifert, Secher, Grøndal, & van Hall, 2015; Nair, Welle, Halliday, & Campbell, 1988). During moderate intensity exercise, infusion of sodium AcAc after an overnight fast attenuates the rise in plasma lactate (Féry & Balasse, 1988), whereas sodium βHB infusion similarly alters the metabolic response to very intense exercise in rats (Kamysheva & Ostrovskaia, 1980) and ischemic forearm exercise in humans (Lestan, Walden, Schmaltz, Spychala, & Fox, 1994).

Despite these observations, the potential performance benefits of ketone bodies have been unexplored until the recent emergence of exogenous ketone supplements in the form of ketone esters and ketone salts. For instance, acute ingestion of the *(R)*-3-hydroxybutyl *(R)*-3-hydroxybutyrate ketone monoester produced plasma βHB concentrations of ~3 mM during exercise and improved 30 min time-trial performance by 2% in elite cyclists (Cox et al., 2016). Ketone esters are not commercially available to date, but ketone salts represent a cheaper, more readily-available exogenous ketone supplement. These salts comprise of the free acid form of βHB buffered with sodium, potassium, and/or calcium salts but are less effective at elevating plasma βHB concentrations compared to the ketone monoester (Stubbs et al., 2017). The effect of acute ketone salt ingestion on short-duration, high-intensity exercise performance in humans has been the subject of two recent reports (O'Malley, Myette-Cote, Durrer, & Little, 2017; Rodger, Plews, Laursen, & Driller, 2017), both of which did not observe the performance benefits associated with the ketone monoester. Given that this is an emerging field of research and to better understand the impact of ketone salt ingestion on responses across a range of exercise intensities, the present study investigated the effect of acute ingestion of a commercially available βHB salt formulation on the metabolic and physiological responses to a graded submaximal exercise session in young, trained male and female cyclists.

Methods

Participants

Nineteen trained cyclists (12 male, 7 female; Table I) gave written informed consent to participate after written and verbal explanation of the procedures. Ethical approval (permit number: LS-15-82-Evans-Egan) was obtained from the University College Dublin Research Ethics Committee. All participants were active in regular cycling training (≥6 sessions per week) and competition in road, time-trial, and/or triathlon disciplines and had been competing in their respective discipline for at least one calendar year.

Experimental design

Participants visited the laboratory for exercise tests on three separate occasions. All tests were performed on the same electronically braked stationary cycle

Table I. Participant anthropometrics and fitness profile.

	Whole cohort ($n = 19$)	Males ($n = 12$)	Females ($n = 7$)	Males vs. females, p value
Age (y)	26.8 ± 7.6	25.6 ± 6.4	30.6 ± 8.6	.148
Height (m)	174.3 ± 8.9	178.7 ± 7.4	166.7 ± 5.7	.002
Body mass (kg)	69.0 ± 9.7	73.8 ± 6.8	60.9 ± 8.5	<.001
Body fat (%)	17.6 ± 6.8	13.7 ± 3.9	24.3 ± 5.0	<.001
FFM (kg)	57.7 ± 10.5	64.3 ± 5.9	46.4 ± 5.3	<.001
W_{max} (W)	325 ± 67	368 ± 40	251 ± 23	<.001
LT (W)	245 ± 59	278 ± 41	187 ± 36	<.001
VO_{2peak} (L min^{-1})	4.27 ± 0.85	4.83 ± 0.43	3.33 ± 0.43	<.001
VO_{2peak} (mL kg^{-1} min^{-1})	61.6 ± 7.1	65.5 ± 5.6	54.9 ± 3.6	<.001
VO_{2peak} (mL kg FFM^{-1} min^{-1})	74.0 ± 5.1	75.2 ± 5.1	71.8 ± 4.7	.171

Note: Data are presented as mean ± SD. LT, power output at 4 mM lactate threshold; FFM, fat-free mass.

ergometer (Lode Excalibur Sport, Netherlands). Saddle height and handlebar position were adjusted to each participant's preference, but kept consistent for the three visits. Participants performed the exercise tests in their own cycling shoes with appropriate pedals provided by the laboratory. Body mass and height were measured using digital scales (SECA, Germany) and a wall-mounted stadiometer (Holtain, UK), respectively. Body composition was measured using dual-energy X-ray absorptiometry (Lunar iDXA, GE Healthcare, UK).

During their first visit to the lab, participants performed a submaximal incremental exercise test to establish their lactate threshold, after which they performed an incremental exercise test to volitional exhaustion to establish their peak oxygen uptake (VO_{2peak}). Two experimental trials, each comprised of a graded exercise test of six stages (at power outputs corresponding to approximately 30%, 40%, 50%, 60%, 70%, and 80%VO_{2peak}), with each stage lasting 8 min (Figure 1), were performed during subsequent visits in a randomized cross-over design. Each experimental trial was identical with the exception of a drink consumed in the hour prior to each exercise test, namely plain water (CON), or βHB salts (KET).

Incremental exercise tests

Assessment of lactate threshold and VO_{2peak} was performed in accordance with guidelines from the British Association of Sport and Exercise Sciences (BASES) (Davison & Wooles, 2007; Spurway & Jones, 2007). Briefly, for the determination of lactate threshold, participants completed 4 min stages (3 min of cycling and 1 min of rest), starting at 50 W. The power output was increased by 50 W for the next two stages, and 30 W thereafter until a blood lactate concentration (Lactate Pro 2, Japan) of 4 mM was exceeded. After a 15 min rest, VO_{2peak} was determined via an incremental test to exhaustion. Participants began cycling at a pre-determined power output based on body mass as per the BASES guidelines, and power output was progressively increased by 20 W min^{-1} for males and 15 W min^{-1} for females thereafter until volitional exhaustion.

Pre-trial preparation

All experimental trials were performed between 07:00 and 10:00, but on an individual basis, participants performed their second trial at the same time ±1 h as their first trial. Pre-trial preparation was the same for each experimental trial. Participants were asked to abstain from alcohol for 48 h and caffeine for at least 12 h and refrain from strenuous exercise training for the day prior to each trial. Each trial took place after a standardized 10 h overnight fast. Participants were asked to keep a one-day portion estimate food diary for the day corresponding to two days prior to the first trial. They were instructed to repeat this pattern of intake before their second trial. On the day immediately prior to both experimental trials, participants were provided with a standardized diet (Gourmet Fuel, Ireland), which provided 40 kcal kg body $mass^{-1}$ at a macronutrient ratio of 40% carbohydrate, 30% protein, and 30% fat. Male participants performed the two experimental trials separated by 7 or 14 days. Because the phase of the menstrual cycle influences fuel utilization during exercise (Oosthuyse & Bosch, 2010), female participants performed the two experimental trials separated by 7 days, but within the early to mid-luteal phase of their menstrual cycle.

Experimental trials

Experimental trials were performed in a randomized cross-over open-label design and were identical with the exception of the drink consumed in the hour prior to each exercise test. The open-label design was chosen because of the difficulty in masking the pungent taste of the βHB salts and considered acceptable because there was no performance element to the experimental design. Therefore, neither the study participants, nor research personnel were blinded to allocation of condition, with the exception of the laboratory technician who did undertake analysis of the blood samples in a blinded manner.

During each trial, a bolus of a given drink was ingested at both 60 min prior to and 15 min prior to the commencement of exercise (Figure 1). Each bolus consisted of either (i) plain water provided at 3.8 mL kg body $mass^{-1}$ (CON), or (ii) βHB salts (KetoCaNa, Prototype Nutrition, IL USA) provided at 0.38 g kg body $mass^{-1}$ dissolved in 3.8 mL kg body $mass^{-1}$ plain water (KET). Each bolus serving of KET provided ~18.5 g βHB, 2.1 g sodium and 1.8 g calcium, which is approximately 60% more βHB than the manufacturer's guidelines of 11.7 g βHB per serving. This timing and dosing strategy was based on our own pilot experiments (unpublished data) showing that plasma βHB concentration peaked at 60 min after ingestion of a single bolus, and that a greater elevation in plasma βHB concentration could be achieved with two smaller doses of βHB salts compared with a single larger dose equivalent to the same total amount of βHB salts.

Upon arrival at the laboratory, an indwelling catheter was introduced into an antecubital vein for

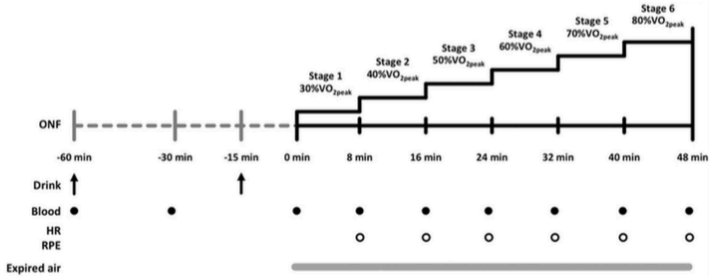

Figure 1. Experimental design schematic. After an overnight fast (ONF), test drinks [water (CON) and βHB salts (KET)] were consumed in two boluses at 60 and 15 min prior to exercise. The graded exercise test consisted of six stages of 8 min in duration performed on a stationary electronically-braked cycle ergometer. HR, RPE, and venous blood were sampled in the last 30 s of each stage. Expired air was collected continuously throughout.

serial blood sampling at rest (−60, −30, and 0 min) and during exercise (last 30 s of each 8 min stage) (Figure 1). The catheter was maintained patent between samples with saline (0.9% sodium chloride). The exercise test was graded and consisted of six stages at power outputs corresponding to approximately 30%, 40%, 50%, 60%, 70%, and 80% VO_{2peak}, with each stage lasting for 8 min (Figure 1). Expired air was collected continuously throughout each exercise test on a breath-by-breath basis (COSMED Quark b2, Italy). During the last 30 s of each 8 min stage, heart rate (HR; Polar, Finland) and rating of perceived exertion (RPE; Borg scale) were recorded, and a blood sample was collected for measurement of plasma βHB, lactate, and glucose concentrations.

Blood analysis

Blood samples (4 mL) were collected in plastic tubes containing sodium fluoride/potassium oxalate (Vacuette Glucose tubes, Greiner-Bio-One, Germany) for subsequent analysis. Samples were stored on ice before centrifugation at 3000*g* for 10 min at 4°C, after which three aliquots of plasma were separated for storage at −80°C until later analysis of plasma βHB, lactate, and glucose (RX Daytona, Randox Laboratories, UK; assay codes RB1007, LC2389, and GL364, respectively).

Data analysis

Cardiopulmonary and metabolic parameters. Minute ventilation (V_E), VO_2, carbon dioxide production (VCO_2), and RER were calculated from an average of breath-by-breath measurements during the last 30 s of each stage in the incremental exercise tests and during the last 2 min of each stage in main experimental trials. Oxygen pulse (O_2 pulse), defined as oxygen uptake per heartbeat and expressed in mL beat^{-1}, was calculated by dividing VO_2 (L min^{-1}) by HR (beats min^{-1}) during the last 30 s of each stage.

Substrate utilization. The rate of energy expenditure (kcal min^{-1}) during each stage was calculated from average VO_2 and VCO_2 values during the last 30 s of each stage using equations applied on an intensity-dependent basis (Jeukendrup & Wallis, 2005). Rates of carbohydrate and fat oxidation are not reported because of the likely error introduced into these calculations by the oxidation of βHB and AcAc, which yield respiratory quotient values of 0.89 and 1.00, respectively (Frayn, 1983). Therefore, reporting oxidation rates based on RER is inaccurate during periods of nutritional ketosis unless appropriate correction factors for CO_2 displacement and excretion of ketone bodies in urine and expired air are employed (Frayn, 1983), which were beyond the scope of the current work.

Mechanical efficiency. Gross efficiency (GE) was calculated as the ratio of the work performed per minute (W converted to kJ min^{-1}) to the energy expended per minute (kJ min^{-1}) at each stage, expressed as a percentage. Delta efficiency (DE) was calculated as the ratio of the change in work performed per minute to the change in energy expended per minute between each stage, expressed as a percentage (Gaesser & Brooks, 1975).

Statistical analysis

Data were evaluated using GraphPad Prism 6 (GraphPad Software, Inc., CA, USA), and are presented as mean ± SD, with the exception of Figure 2 where

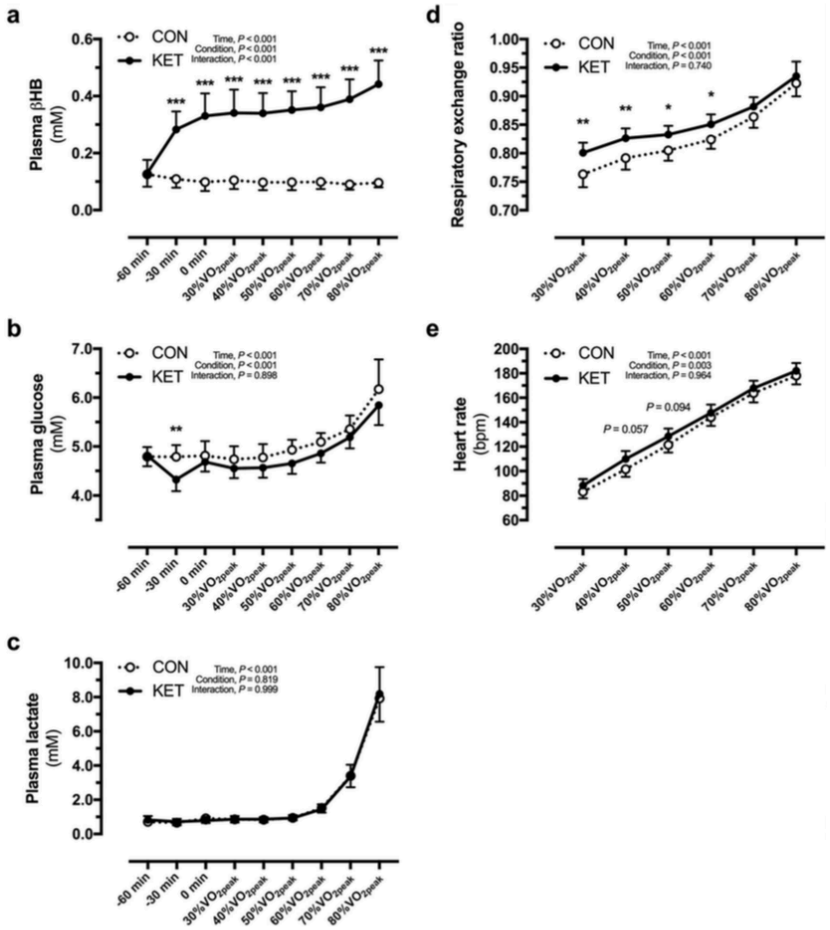

Figure 2. Plasma βHB (a), glucose (b) and lactate (c) concentrations, respiratory exchange ratio (d), and HR (e) during graded exercise on a cycle ergometer in a fasted state (CON) or after ingestion of βHB salts (KET). Data are presented as mean values, with error bars representing 95% confidence intervals. *$p < .05$ KET vs. CON; **$p < .01$ KET vs. CON; ***$p < .001$ KET vs. CON.

error bars represent 95% confidence intervals. The experiment was powered based on change in RER as the primary outcome, which was chosen as a measure of an altered metabolic response. Based on the aforementioned pilot data where a 0.034 ± 0.015 difference in RER between KET and CON was observed, $n = 13$ participants would have been required given $\alpha = 0.05$ and $1 - \beta = 0.8$ (GPower v3.1). Independent samples *t*-tests were used to determine differences between male and female participants for baseline characteristics. Two-way (condition × intensity) repeated measures analysis of variance was used to determine differences between the two experimental trials for variables with serial measurements. When a main effect of condition, or an interaction effect between condition and intensity, was indicated, post-hoc testing was performed using Holm–Sidak's multiple comparisons test with multiplicity-adjusted *p* values to compare KET to CON at respective time points. The data were tested for normality and sphericity prior to proceeding with the tests described. In addition, standardized differences in the mean were used to assess magnitudes of effects between conditions at respective time points.

These were calculated using Cohen's *d* effect size (ES) and interpreted using thresholds of <0.25, >0.25, >0.5, and >1.0 for trivial, small, moderate, and large, respectively (Rhea, 2004). Pearson's product–moment correlation coefficient (*r*) was used to explore correlations between variables. No differences were observed between male and female participants for the effect of KET on the metabolic response to exercise compared to water, so male and female data are presented as combined ($n = 19$) data unless otherwise stated. The significance level was set at $\alpha = 0.05$ for all statistical tests.

Results

Plasma βHB, glucose and lactate

Fasting plasma βHB (KET, 0.13 ± 0.10 mM; CON, 0.12 ± 0.09 mM; ES = .05) and glucose (KET, 4.82 ± 0.46 mM; CON, 4.79 ± 0.40 mM; ES = .06) concentrations did not differ between trials (Figure 2). Ingestion of KET resulted in a rise in plasma βHB concentration to 0.28 ± 0.13 mM ($p < .001$) 30 min after ingestion and remained elevated throughout exercise ($p < .001$) (Figure 2(a)). The highest plasma βHB concentration during KET was observed in the final stage of exercise at 0.44 ± 0.15 mM ($p < .001$). Plasma glucose concentration averaged 0.44 ± 0.27 mM lower 30 min after ingestion of KET compared to CON ($p = .008$; ES = .96). An inverse correlation ($r = -0.647$, $p = .004$) was observed for the change in plasma βHB and glucose concentrations at this time point. Plasma glucose concentrations remained lower at all stages throughout exercise, with ES indicating small to moderate effects, i.e. 30%VO_{2peak}, −0.19 ± 0.36 mM, ES = .39; 40%,VO_{2peak}, −0.21 ± 0.43 mM, ES =.44; 50%VO_{2peak}, −0.27 ± 0.40 mM, ES = .66; 60% VO_{2peak}, −0.21 ± 0.39 mM, ES = .62; 70%VO_{2peak}, −0.17 ± 0.54 mM, ES = .33; and 80%VO_{2peak}, −0.39 ± 1.24 mM, ES = .34 (Figure 2(b)). Plasma lactate concentrations were elevated above resting values during the final two stages of exercise, but no differences between KET and CON were observed for plasma lactate concentrations at any time point (Figure 2(c)).

Cardiorespiratory responses to graded exercise and KET ingestion

All cardiorespiratory parameters exhibited main effects for exercise intensity (all $p < .001$). No differences in %VO_{2peak}, VO_2, VCO_2, or V_E were observed between conditions (Table II). RER was elevated by KET ($p < .001$ for condition), and was ~0.03 higher for intensities up to 60%VO_{2peak} (all $p < .05$), with ES indicating moderate effects at these intensities, i.e. 30%VO_{2peak}, 0.038 ± 0.030, $p = 0.003$, ES = .90; 40%,VO_{2peak}, 0.035 ± 0.036, $p = 0.007$, ES = .92; 50%VO_{2peak}, 0.028 ± 0.031, $p = 0.025$, ES = .81; and 60%VO_{2peak}, 0.027 ± 0.037, $p = 0.031$, ES = .78 (Figure 2(d)). The effect of KET on RER was small at 70%VO_{2peak} (0.018 ± 0.030, $p = 0.16$, ES = .50) and 80%VO_{2peak} (0.012 ± 0.045, $p = 0.37$, ES = .28). HR was also elevated by KET ($P = .003$ for condition), wherein HR averaged ~4 to 8 bpm higher during KET and ES indicated small to moderate effects, i.e. 30%VO_{2peak}, 5.6 ± 4.5 bpm, ES = .48; 40%,VO_{2peak}, 8.5 ± 7.1 bpm, ES = .66; 50%VO_{2peak}, 7.8 ± 7.1 bpm, ES = .55; 60%VO_{2peak}, 3.9 ± 8.6, ES = .26; 70%VO_{2peak}, 4.9 ± 8.2 bpm, ES = .29; and 80%VO_{2peak}, 4.4 ± 7.0 bpm, ES = .34 (Figure 2(e)). No differences in oxygen pulse, RPE, GE, or DE were observed between conditions (Table II).

Gastrointestinal responses

Thirteen out of 19 (68%) participants reported symptoms of gastrointestinal distress in response to KET ingestion. These comprised of seven (37%), three (16%), two (11%), and one (5%) of the participants reporting nausea, diarrhoea, vomiting, and lightheadedness, respectively. These symptoms manifested in the latter stages of and immediately after the cessation of exercise. No symptoms were reported during CON.

Discussion

The aim of the present study was to investigate the effect, if any, of acute ingestion of βHB salts on metabolic and physiological responses to a graded exercise session in trained cyclists. Ingestion of commercially available βHB salts resulted in elevated plasma βHB concentrations (>0.3 mM) at rest and during exercise. This coincided with elevated RER (moderate effects) and HR (small to moderate effects) during submaximal exercise intensities, and a lowering of plasma glucose concentrations (small to moderate effects), compared with the ingestion of water. However, a range of other parameters including plasma lactate, rate of perceived exertion, GE, and DE were unaffected by the acute ingestion of βHB salts.

Exogenous ketone supplements, such as βHB salts, represent a novel method to increase the concentration of circulating ketone bodies without implementing restrictive dietary practices such as fasting or low carbohydrate, ketogenic diets (Cox & Clarke, 2014; Evans et al., 2017). Despite the increasing

Table II. Cardiorespiratory responses during graded exercise in CON or KET.

	Stage 1 30% VO_{2peak}	Stage 2 40% VO_{2peak}	Stage 3 50% VO_{2peak}	Stage 4 60% VO_{2peak}	Stage 5 70% VO_{2peak}	Stage 6 80% VO_{2peak}	Intensity, *p* value	Condition, *p* value	Interaction, *p* value
$\%W_{max}$									
KET	14 ± 6	26 ± 6	38 ± 5	50 ± 5	62 ± 4	74 ± 5	<.001	>.999	>.999
CON	14 ± 6	26 ± 6	38 ± 5	50 ± 5	62 ± 5	74 ± 5			
$\%VO_{2peak}$									
KET	27 ± 4	38 ± 4	49 ± 4	61 ± 4	73 ± 5	85 ± 5	<.001	>.999	>.999
CON	27 ± 4	37 ± 4	49 ± 3	61 ± 3	73 ± 5	85 ± 4			
V_E ($L\,min^{-1}$)									
KET	26.6 ± 7.6	36.0 ± 8.9	46.4 ± 10.4	59.2 ± 12.7	79.3 ± 18.8	109.5 ± 30.4	<.001	.676	>.999
CON	25.3 ± 7.5	35.0 ± 9.1	45.9 ± 10.1	58.4 ± 12.5	78.4 ± 18.0	108.6 ± 30.8			
ES	.18	.12	.05	.06	.05	.03			
VO_2 ($L\,min^{-1}$)									
KET	1.21 ± 0.35	1.65 ± 0.40	2.12 ± 0.45	2.59 ± 0.54	3.15 ± 0.63	3.63 ± 0.72	<.001	.954	>.999
CON	1.18 ± 0.35	1.63 ± 0.42	2.12 ± 0.45	2.61 ± 0.56	3.17 ± 0.67	3.66 ± 0.78			
ES	.08	.04	.01	.03	.03	.03			
VCO_2 ($L\,min^{-1}$)									
KET	0.97 ± 0.30	1.37 ± 0.35	1.77 ± 0.41	2.21 ± 0.49	2.79 ± 0.61	3.41 ± 0.78	<.001	.399	>.999
CON	0.90 ± 0.29	1.29 ± 0.35	1.71 ± 0.38	2.15 ± 0.46	2.74 ± 0.61	3.38 ± 0.74			
ES	.23	.20	.16	.14	.08	.04			
RPE									
KET	6.4 ± 0.5	8.1 ± 1.2	10.1 ± 1.8	12.4 ± 1.9	14.4 ± 2.1	16.5 ± 2.3	<.001	.969	>.999
CON	6.4 ± 1.0	8.4 ± 1.7	10.9 ± 1.9	13.1 ± 1.3	14.9 ± 1.1	17.3 ± 1.5			
ES	.07	.23	.45	.43	.30	.38			
O_2 pulse ($mL\,beat^{-1}$)									
KET	13.3 ± 3.5	14.7 ± 3.4	16.3 ± 3.1	17.3 ± 3.4	18.6 ± 3.5	19.4 ± 3.9	<.001	.075	.992
CON	14.0 ± 3.6	16.0 ± 3.8	17.5 ± 3.7	18.2 ± 4.1	19.3 ± 4.5	20.6 ± 4.7			
ES	.20	.36	.36	.23	.17	.26			
GE (%)									
KET	10.4 ± 3.6	15.0 ± 2.3	16.9 ± 1.9	18.2 ± 1.7	18.8 ± 1.6	19.1 ± 1.9	<.001	.789	.992
CON	10.7 ± 3.6	15.2 ± 2.0	16.9 ± 1.7	18.2 ± 1.1	18.8 ± 1.0	19.2 ± 1.0			
ES	.07	.10	.01	.04	.03	.12			
DE (%)									
KET	N/A	27.0 ± 4.7	23.1 ± 3.4	24.7 ± 5.7	21.6 ± 3.0	19.9 ± 6.8	<.001	.737	.830
CON	N/A	28.2 ± 9.6	22.7 ± 3.9	24.2 ± 5.2	20.9 ± 5.9	21.8 ± 7.0			
ES		.16	.12	.09	.16	.28			

Notes: Data are presented as mean ± SD. ES was calculated as Cohen's *d* and interpreted using thresholds of <0.25, >0.25, >0.5, and >1.0 for trivial, small, moderate, and large, respectively. $\%W_{max}$, percentage of maximum power output; $\%VO_{2peak}$, percentage of peak oxygen uptake; DE, delta efficiency; GE, gross efficiency; V_E, minute ventilation; VO_2, rate of oxygen uptake; VCO_2, rate of carbon dioxide production; RPE, rating of perceived exertion.

commercial availability of βHB salts, to date, there is a paucity of data from humans on the metabolic response to ingestion at rest or during exercise. The sodium/potassium βHB mineral salt ingested in the present study resulted in a modest elevation (~0.3 to 0.4 mM) in plasma βHB concentrations. These values are similar to those observed after a 24 h fast (Balasse & Féry, 1989; Laffel, 1999) and can be considered to have produced nutritional ketosis (i.e. >0.2 mM (Robinson & Williamson, 1980). The dosing strategy employed involved a bolus ingested both at 60 min and 15 min prior to exercise, but plasma βHB concentrations peaked during the last stage of exercise at 0.44 ± 0.15 mM. This suggests that the supplement was still being released into circulation approximately one hour after the ingestion of the second bolus, a time course consistent with several recent reports describing βHB salt ingestion at rest (Stubbs et al., 2017) and prior to exercise (O'Malley et al., 2017; Rodger et al., 2017). However, these studies reported somewhat higher blood βHB concentrations (~0.6 to 1.0 mM) after ingestion of doses providing of 2× 11.7 g of βHB (Rodger et al., 2017), ~21 to 27 g of βHB (O'Malley et al., 2017), and ~12 g or ~25 g of βHB (Stubbs et al., 2017), compared to the 2× ~18.5 g in the present study. However, unlike the present study, these studies measured βHB concentrations in whole blood from finger-prick sampling using handheld devices, which are known to overestimate blood βHB concentration ranging from 50% to three-fold relative to lab-based measures performed on serum (Guimont et al., 2015; Leckey, Ross, Quod, Hawley, & Burke, 2017).

The aim of ingestion of exogenous ketone supplements is to achieve acute nutritional ketosis (Cox & Clarke, 2014), and this is readily-achieved by the *(R)*-3-hydroxybutyl *(R)*-3-hydroxybutyrate ketone monoester (Cox et al., 2016). Ingestion of 573 mg kg body mass^{-1} of that supplement raises plasma βHB concentrations to ~3 mM 10 min after ingestion, which rise further to ~6 mM within the next 60 min at rest (Cox et al., 2016). Clearly the βHB salts ingested in the present study produce plasma βHB concentrations that are ~10-fold less than this. Despite the modest change in plasma βHB concentrations, the acute ingestion of βHB salts does exert some metabolic action at rest and during exercise. For instance, a ~10% decline in plasma glucose was observed 30 min after the ingestion of βHB salts, with an inverse correlation observed between the respective changes in plasma βHB and glucose concentrations at this time. This is consistent with the acute infusion of ketone bodies producing βHB concentrations of ~0.5 to 1 mM resulting in a decline in plasma glucose of ~10% (Mikkelsen et al., 2015; Sherwin, Hendler, & Felig, 1975), and similar results associated with βHB salt ingestion (Stubbs et al., 2017). Moreover, a slightly lower plasma glucose concentration (~0.2 mM; small to moderate effects) was evident throughout exercise in the present study, which confirms other recent reports (Leckey et al., 2017; O'Malley et al., 2017; Rodger et al., 2017).

Other effects observed during exercise in the present study include elevations in RER (moderate effects) and HR (small to moderate effects) during the low-to-moderate intensities of exercise. The elevation in RER may be indicative of oxidation of ketone bodies during exercise based on the stoichiometry of oxidation of AcAc. Before being oxidized as a fuel source in skeletal muscle, βHB is re-oxidized to AcAc through the action of 3-hydroxybutyrate dehydrogenase (BDH). The respiratory quotient for oxidation of AcAc (1.0) is identical to that glucose (Frayn, 1983). Therefore, a contribution of ketone oxidation to energy provision likely explains the elevation in RER during exercise after ingestion of βHB salts in the present study. The elevated RER is consistent with a recent report of prolonged submaximal exercise in trained male cyclists (Rodger et al., 2017), but the opposite of what was reported during graded exercise in recreationally active men (O'Malley et al., 2017). Like the former study, we studied trained cyclists, so whether training status is the only explanation for the divergent findings remains to be confirmed. However, this would be consistent with our previous suggestion that oxidation of ketone bodies during exercise is likely to be greatest in trained participants with a high proportion of type I muscle fibres and/or a high oxidative capacity in skeletal muscle (Evans et al., 2017).

Calculations of arteriovenous differences of radiolabelled ketone bodies across working muscles estimate the contribution of ketone bodies to energy provision of 2–10% during exercise in the fasted state (Balasse et al., 1978; Féry & Balasse, 1983; Wahren et al., 1984). This contribution is unlikely to be >10% unless plasma βHB concentrations are elevated above 1 mM and exercise is being performed by trained participants (Evans et al., 2017). In well-trained participants consuming exogenous ketones as a ketone monoester, the contribution of ketone bodies to energy provision is greater, i.e. 16–18% of total oxygen consumption (Cox et al., 2016). Therefore, although the elevation in plasma βHB concentration in the present study was modest, it is likely that this did result in an increased contribution of ketone bodies to energy provision during exercise.

Apart from a contribution to energy provision, the principal efficacy of supplemental ketones as a performance aid is likely to be secondary effects on

metabolism and alterations in fuel selection (Evans et al., 2017). For instance, acute infusion of sodium AcAc (Féry & Balasse, 1988) or sodium βHB (Lestan et al., 1994) attenuates the exercise-induced rise in plasma lactate, an effect also observed after ingestion of the aforementioned ketone monoester (Cox et al., 2016). In the latter work, reduced glycolytic flux, glycogen sparing, and increased contribution of intramuscular triglyceride to energy provision were observed during 2 h of cycling at ~70% VO_{2max}. However, an attenuation of the rise in plasma lactate was not observed in the present study or in other recent studies examining acute ingestion of βHB salts (O'Malley et al., 2017; Rodger et al., 2017). Again, this might be explained by the relatively lower increase in plasma βHB concentration produced by the βHB salts compared to the ketone monoester.

An important methodological note is that the βHB salts used in the present study provide a racemic mixture of βHB, i.e. containing both the D- and L-enantiomers of βHB (also designated *R*- and *S*-, respectively), whereas the βHB assay employed determines the concentration of D-βHB. D-βHB is the circulating and primary form of βHB (Tsai et al., 2006), but intracellular concentrations of L-βHB are sensitive to factors such as aging and metabolic health (Hsu et al., 2011). The D- and L- enantiomers of βHB exert divergent physiological effects on glucose metabolism in the heart (Tsai et al., 2006) and skeletal muscle (Yamada, Zhang, Westerblad, & Katz, 2010), and on longevity (Edwards et al., 2014). Recent work has demonstrated that racemic βHB ingested as βHB salts results in elevations in L-βHB concentrations of ~2 mM (Stubbs et al., 2017). However, it is doubtful that a change in circulating L-βHB concentration, if provided by an exogenous ketone supplement, would have any direct effect on substrate metabolism in skeletal muscle. For instance, L-βHB is not a substrate for mitochondrial BDH and thus is not metabolized to AcAc (Scofield et al., 1982), and its physiological role is most likely in the synthesis of sterols and fatty acids in non-muscle tissues (Webber & Edmond, 1977).

The small to moderate effects observed for an elevated HR of 4–8 bpm after ingestion of βHB salts compared to water may warrant future investigation. HR during the exercise was not reported in previous work with βHB infusion, or ketone monoester or βHB salt ingestion, but was elevated by 25% under resting conditions after sodium βHB infusion compared to saline infusion (Gormsen et al., 2017). This indicates an effect of βHB itself rather than sodium load, but occurred at a plasma βHB concentration of ~4 mM in contrast to ~0.4 mM in the present study. Alternatively, the sodium load delivered by the βHB salts may exert some hemodynamic effects. Acute sodium ingestion can transiently elevate blood pressure (Farquhar, Paul, Prettyman, & Stillabower, 2005), and sodium bicarbonate ingestion providing a similar dose of sodium to the present study results in an elevation in HR of ~10 bpm during moderate intensity exercise (Kahle, Kelly, Eliot, & Weiss, 2013).

Also notable in the present study was that 13 out of 19 (68%) participants reported symptoms of gastrointestinal distress after exercise in the βHB salt condition. The hypertonic nature of the βHB salts ingested likely caused an intraluminal osmotic load and water shift into the intestinal lumen resulting in osmotic diarrhoea. However, gastrointestinal distress is also a potential side effect of acute ingestion of ketone esters, with high prevalence noted after the ingestion of the ketone diester by elite cyclists (Leckey et al., 2017), and increasing incidences occurring with increasing dosages of the ketone monoester (Clarke et al., 2012). Clearly, such issues would be deleterious to exercise performance, and, therefore, require further exploration, either in terms of optimal dosing strategies, or whether repeat exposure to exogenous ketone supplements reduces these symptoms.

In conclusion, acute ingestion of a commercially available βHB salt formulation by trained cyclists resulted in a modest increase in plasma βHB concentrations before and during graded exercise to levels that can be considered acute nutritional ketosis. This resulted in alterations in the metabolic and physiological response to exercise as evidenced by lowering of plasma glucose concentrations and elevated RER and HR values at low-to-moderate exercise intensities compared to ingestion of water. However, no effect was observed on perceived exertion or muscular efficiency or on plasma lactate concentrations. This is in contrast to previous work using βHB infusion or ingestion of a ketone monoester supplement, both of which achieve markedly higher plasma βHB concentrations during exercise. This suggests the likelihood that a dose-response effect exists for exogenous ketone supplements on metabolic responses and exercise performance. Given the gastrointestinal issues observed with the present βHB salts, further work is needed with other methods of increasing circulating ketone concentrations including improved free acid or mineral salt formulations, before the merit, if any, of ketone salts for performance enhancement in athletes is likely to be realised.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Appendix C: Published Medicine and Science in Sport and Exercise article

Intermittent Running and Cognitive Performance after Ketone Ester Ingestion

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ABSTRACT

EVANS, M., and B. EGAN. Intermittent Running and Cognitive Performance after Ketone Ester Ingestion. *Med. Sci. Sports Exerc.*, Vol. 50, No. 11, pp. 2330–2338, 2018. **Purpose**: Ingestion of exogenous ketones alters the metabolic response to exercise and may improve exercise performance, but it has not been explored in variable-intensity team sport activity, or for effects on cognitive function. **Methods**: On two occasions in a double-blind, randomized crossover design, 11 male team sport athletes performed the Loughborough Intermittent Shuttle Test (part A, 5 × 15-min intermittent running; part B, shuttle run to exhaustion), with a cognitive test battery before and after. A 6.4% carbohydrate–electrolyte solution was consumed before and during exercise either alone (PLA) or with 750 $mg \cdot kg^{-1}$ of a ketone ester (KE) supplement. Heart rate, RPE, and 15-m sprint times were recorded throughout, and serial venous blood samples were assayed for plasma glucose, lactate, and β-hydroxybutyrate. **Results**: KE resulted in plasma β-hydroxybutyrate concentrations of ~1.5 to 2.6 mM during exercise ($P < 0.001$). Plasma glucose and lactate concentrations were lower during KE compared with PLA (moderate-to-large effect sizes). Heart rate, RPE, and 15-m sprint times did not differ between trials. Run time to exhaustion was not different ($P = 0.126$, $d = 0.45$) between PLA (mean = 268 s, 95% confidence interval [CI] = 199–336 s) and KE (mean = 229 s, 95% CI = 178–280 s). Incorrect responses in a multitasking test increased from pre- to postexercise in PLA (mean = 1.8, 95% CI = −0.6 to 4.1) but not in KE (mean = 0.0, 95% CI = −1.8 to 1.8) ($P = 0.017$, $d = 0.70$). **Conclusion**: Compared with carbohydrate alone, coingestion of a KE by team sport athletes attenuated the rise in plasma lactate concentrations but did not improve shuttle run time to exhaustion or 15-m sprint times during intermittent running. An attenuation of the decline in executive function after exhausting exercise suggests a cognitive benefit after KE ingestion. **Key Words**: β-HYDROXYBUTYRATE, LACTATE, LOUGHBOROUGH INTERMITTENT SHUTTLE TEST, RUNNING, TEAM SPORT

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Ketone bodies, namely, β-hydroxybutyrate (βHB), acetoacetate, and acetone, are fatty acid metabolites whose production markedly increases in physiological states characterized by reduced glucose availability, such as starvation and ketogenic diets (1,2). Ketone bodies are principally produced as a survival mechanism to provide a substrate for the brain, but they are also oxidized by skeletal muscle and provide up to 10% of energy during exercise in a fasted state (3). Infusion of ketone bodies exerts a range of metabolic actions, such as attenuation of hepatic glucose output, antilipolytic effects in adipose tissue and glucose "sparing," and stimulation of protein synthesis in skeletal muscle (4–6).

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The effects of ketone bodies on substrate utilization during exercise and, consequently, athletic performance are of increasing interest because of the development of exogenous ketone supplements, namely, ketone salts and ketone esters (7–9). These formulations represent a method of acutely inducing nutritional ketosis (plasma βHB >0.5 mM) resulting in a variety of effects on exercise metabolism, performance, and recovery (10–17). Ketone salts in their presently available racemic form produce only modest changes (<1.0 mM) in plasma βHB concentrations (9,12,14,16,17). Although their preexercise ingestion can alter the metabolic response to exercise (12,16), there is no evidence of an ergogenic effect (12,14). Alternatively, exogenous ketone supplements in the form of ketone esters produce markedly greater changes in plasma βHB concentrations than ketone salts in humans (9) and rats (8).

Two ketone esters have been reported in the recent literature: a R,S-1,3-butanediol acetoacetate ketone diester (KDE) (8,15) and a (R)-3-hydroxybutyl (R)-3-hydroxybutyrate ketone monoester (KME) (7,9,10). Both esters were tested in elite endurance athletes with divergent findings (10,15). Acute ingestion of KME produced plasma βHB concentrations of ~3.0 mM after 20 min and improved 30 min time-trial performance by 2% (10). By contrast, acute ingestion of KDE was less effective at raising serum βHB concentrations (~0.4 mM) and impaired 31.2-km time-trial performance by 2% (15). Consumption of

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KME increased the contribution of ketone bodies to fuel provision during exercise to 16%–18% of total energy provision, in addition to marked metabolic effects, including the attenuation of blood lactate concentrations, "sparing" of muscle glycogen, and increased intramuscular triglyceride utilization (10). The reduction in glycolytic flux may reflect an impairment of carbohydrate utilization rather than glycogen sparing (18–20), a key question that requires further investigation. The former is likely to impair performance in high-intensity sports that demand a high rate of ATP provision from carbohydrate sources (18).

Team sports such as Australian football, soccer, Gaelic games, rugby union, lacrosse, and field hockey are high-intensity and intermittent in nature, consisting of repeated periods of high-intensity activity (sprinting) interspersed with exercise at low-to-moderate intensities (walking and jogging) (21,22). Nutrition guidelines for soccer, for instance, recommend high intakes of carbohydrate before and during competition to maximize muscle glycogen stores with the view to enhancing performance (23). Soccer match play results in a marked reduction in muscle glycogen, and high-intensity running is attenuated in the last 15 min of play (24,25). Therefore, nutrition strategies that could spare glycogen and maintain high-intensity running in the latter parts of matches are of interest to scientists and practitioners, but research on exogenous ketone supplements to date has mostly focused on athletes from endurance sports (10,14–16). Moreover, because ketone bodies are the dominant fuel source for the brain in ketogenic states (26), there is potential for central and/or cognitive effects of exogenous ketone supplements, but to date cognitive effects were only explored in short-term feeding trials in rats (27–29). Therefore, the aim of the present study was to investigate the effects of acute ingestion of a ketone ester on metabolic responses, physical performance, and cognitive performance in team sport athletes in response to an intermittent running protocol that simulated soccer match play.

METHODS

Participants. Eleven male team sport athletes (mean ± SD: age = 25.4 ± 4.6 yr, height = 1.80 ± 0.05 m, body mass = 78.6 ± 5.3 kg, $\dot{V}O_{2max}$ = 53.9 ± 2.2 mL·kg^{-1}·min^{-1}) gave written informed consent to participate after written and verbal explanation of the procedures. Ethical approval (permit number: DCUREC2017_130) was obtained from the Dublin City University Research Ethics Committee in accordance with the Declaration of Helsinki. All participants were actively training and competing in high-intensity field-based team sports.

Experimental design. Participants visited the laboratory for exercise tests on three separate occasions over a 14- to 21-d period. During their first visit to the laboratory, each participant's maximal oxygen consumption ($\dot{V}O_{2max}$) and speed at $\dot{V}O_{2max}$ were determined using a progressive multistage shuttle run test (Yo-Yo intermittent recovery test level 1; Yo-Yo IR1) (30). These data were used to determine jogging (55% $\dot{V}O_{2max}$) and cruising (95% $\dot{V}O_{2max}$) speeds for use during the Loughborough Intermittent Shuttle Test (LIST). The LIST is a validated simulation of the physiological and metabolic responses during soccer match play and consists of two parts: part A comprises a fixed period of variable-intensity shuttle running over 20 m; part B consists of continuous running, alternating every 20 m between 55% and 95% $\dot{V}O_{2max}$ until volitional fatigue (31). After a 15-min rest after completion of the Yo-Yo IR1, participants were familiarized with the LIST protocol by completing one 15-min block at their personalized running speeds. Cognitive tests were performed before the Yo-Yo IR1 and after familiarization with the LIST to familiarize participants with the cognitive test battery.

Two main experimental trials, each comprising of the LIST (parts A and B) with cognitive tests before and after, were performed during subsequent visits in a double-blinded, randomized crossover design. Both experimental trials included a standardized diet for ~36 h before the exercise test and were identical except for the drinks consumed before and during the LIST, namely, a 6.4% carbohydrate–electrolyte solution, which was either flavored (Symrise, UK) and acted as the control/placebo condition (PLA), or included a ketone ester (KE) (Fig. 1). The primary outcome was endurance capacity measured by run time to exhaustion in the LIST part B, with secondary outcomes including 15-m sprint times during the LIST part A, heart rate (HR), RPE, and plasma glucose, lactate, and βHB concentrations.

Incremental exercise test and familiarization. For determination of $\dot{V}O_{2max}$, jogging (55% $\dot{V}O_{2max}$), and cruising (95% $\dot{V}O_{2max}$) during the LIST, participants completed the Yo-Yo IR1. All participants completed a standardized 5-min warm-up consisting of progressive shuttle runs at 20%, 40%, 60%, and 80% and dynamic stretching (high knees, heel kicks, and groin bridges), followed by a period of self-selected stretching. The Yo-Yo IR1 consists of 40-m shuttle runs (2 × 20 m) between two sets of cones set 20 m apart. Shuttles progressively increase in speed that is dictated by an audio signal (Teambeep Software, UK). Each 40-m shuttle is separated by a 10-s rest period. The test was terminated when participants failed to complete the second 20-m shuttle on two consecutive occasions or if they reached volitional fatigue. $\dot{V}O_{2max}$ was calculated as follows:

$$\dot{V}O_{2max}\ (\text{mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}) = \text{Yo-Yo IR1 distance (m)} \times 0.0084 + 36.4.$$

After a 15-min break, participants were familiarized with the LIST by performing one block of intermittent activity, i.e., 15 min of part A, were allowed 3 min of rest, and then completed the part B run to exhaustion. Participants completed a battery of cognitive tests before the Yo-Yo IR1 and after the intermittent run to exhaustion.

Cognitive test battery. The battery of cognitive tests (CANTAB Cognition, UK) was administered via a touch screen tablet lasting ~25 min. An identical test battery was administered before and after each trial. Technical issues,

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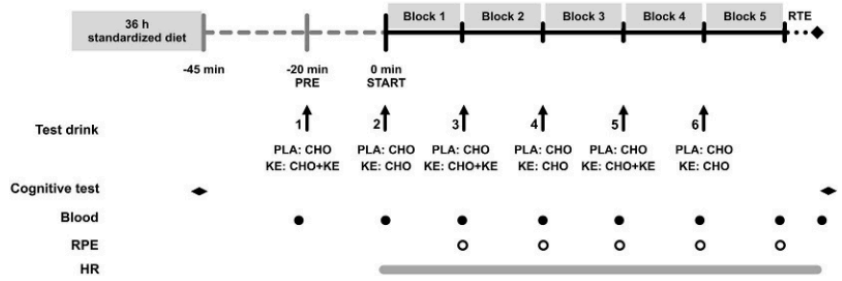

FIGURE 1—Schematic of the study protocol. CHO, carbohydrate–electrolyte solution; PLA, placebo; RTE, shuttle run to exhaustion.

namely, with loss of wireless Internet access during test administration, resulted in the data for the cognitive test battery comprising of $n = 8$ participants.

During the reaction time task, participants select and hold a button at the bottom of the screen, and five circles are presented above. In each case, a yellow dot appears in one of the five circles, and the participant must react as soon as possible, releasing the button at the bottom of the screen and selecting the circle in which the dot appeared. Release time (ms), reaction time (ms), and number of errors were recorded.

The multitasking test is a test of executive function that measures the participant's ability to switch attention between stimuli and ignore task-irrelevant information. White arrows are displayed on a black background, with the arrows located on either the left or right side of the screen, and pointing either to the left or to the right. A cue is displayed at the same time as the arrows, reading either "SIDE" or "DIRECTION." When the "SIDE" cue is presented, the participant is required to press a button on the left or right of the screen corresponding to the side of the screen where the arrow is presented, regardless of the direction the arrow is pointing. Conversely, when the "DIRECTION" cue is presented, the participant is required to touch a button on the left or right of the screen corresponding to the direction the arrow is pointing, regardless of which side of the screen the arrow is presented. Reaction time (ms) and number of correct and incorrect responses were recorded.

The rapid visual information processing task is a test of sustained attention. The participant is presented with a white box in the center of the screen. Single digits ranging from 2 to 9 are presented one at a time in a pseudorandom order inside the box, appearing at a rate of 100 digits per minute. The participant is required to detect specific three-digit sequences, including 2-4-6, 4-6-8, and 3-5-7. As soon as a target sequence is detected, the participant is required to touch a button on the screen. Response latency (ms), correct responses, and false alarms were recorded.

Pretrial preparation. All experimental trials were performed between 3:30 PM and 8:00 PM, but on an individual basis, participants performed their second trial at the same time ±1 h as their first trial. Pretrial preparation was the same for each experimental trial. Participants were asked to abstain from alcohol for 48 h and caffeine for 24 h and refrain from strenuous exercise training the day before each trial. The day before experimental trials, participants were provided with a standardized diet (Gourmet Fuel, Ireland), which provided 40 kcal·kg body mass^{-1} at a macronutrient ratio of 60% carbohydrate, 20% protein, and 20% fat. On the day of experimental trials, participants consumed two meals providing 3 g·kg body mass^{-1} of carbohydrate before arriving at the laboratory. The second meal was consumed 3 h before the initiation of the LIST. In addition to the energy content and macronutrient ratio, the food itself was identical for both trials. Participants performed the two experimental trials separated by either 7 or 14 d.

Experimental trials. Experimental trials were performed in a double-blinded, randomized crossover design and were identical except for the drinks consumed. During each trial, a bolus of a given drink was ingested 20 min before exercise (drink 1), and during each 3-min seated break during part A (drinks 2 to 6) (Fig. 1). During PLA, a 6.4% carbohydrate–electrolyte solution (Lucozade Sport, Lucozade Ribena Suntory Ltd., UK) was provided at a rate of ~1.2 g·min^{-1} of exercise. During KE, a 6.4% carbohydrate–electrolyte solution was provided at a rate of ~1.2 g·min^{-1} combined with 750 mg·kg body mass^{-1} of a R-βHB (R)1,3-butanediol ketone ester (KE4, KetoneAid Inc., Falls Church, VA). The ketone ester was mixed directly with the carbohydrate–electrolyte solution for ingestion in three boluses (50:25:25), i.e., at 20 min before exercise (drink 1), and after 30 (drink 3) and 60 min (drink 5) of exercise, respectively (Fig. 1). During PLA, drinks 1, 3, and 5 were flavored with a bitter additive (Symrise, UK) to taste-match with KE, and in both trials, drinks 2, 4, and 6 were provided as the unadulterated carbohydrate–electrolyte solution. All drinks were administered in opaque drinks bottles.

Upon arrival at the laboratory, participants provided a urine sample for assessment of hydration status (PalOSMO, VITECH Scientific, Japan) and then proceeded to complete the described battery of cognitive tests. Thereafter, an indwelling catheter (21G Insyte Autoguard; Becton Dickinson, Franklin Lakes, NJ) was introduced into an antecubital vein for serial venous blood sampling at rest (−20 and 0 min), during each 3-min seated rest period between the 15-min blocks in part A and immediately after the run to exhaustion.

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Participants were fitted with a Bluetooth HR monitor (Polar V7; Polar Electro Oy, Kempele, Finland) for continuous recording of HR and then performed the standardized 10-min warm up followed by self-selected stretching. Participants then performed the LIST protocol (part A: 5 × 15 min intermittent activity; part B: run to exhaustion) (31). All exercise intensities were based on percentages of $\dot{V}O_{2max}$ determined during the Yo-Yo IR1. The repeating order of activity in part A, which occurs in a continuous manner for each 15 min block, comprises of 3 × 20 m at walking speed, 1 × maximal 15 m sprint, 4-s recovery, 3 × 20 m jogging speed (55% $\dot{V}O_{2max}$), and 3 × 20 m at cruising speed (95% $\dot{V}O_{2max}$). Sprint times were measured by two sets of wireless infrared photoelectric cells (TC Timing System; Brower Timing, Draper, UT).

Part B consists of single 20-m shuttles alternating between jogging (55% $\dot{V}O_{2max}$) and cruising (95% $\dot{V}O_{2max}$) speeds. The shuttle run to exhaustion, measured in seconds, continued until participants were unable to complete two consecutive shuttles at cruising speeds, or until volitional fatigue. All speeds were dictated using audio software (Team Beep Software, UK). All participants received consistent encouragement during the maximal sprinting of part A and the run to exhaustion of part B.

Venous blood samples were collected during the 3-min break between each 15-min block of part A, and RPE (Borg scale) was recorded during the same period. Incidences of gastrointestinal (GI) symptoms were recorded by interview after each trial after completion of the cognitive test battery. After completion of both experimental trials, participants completed an exit interview in which they were asked whether they could identify the KE condition and which trial did they believe that they performed their longest run to exhaustion.

Blood analysis. Blood was collected in plastic tubes (2 mL) containing sodium heparin (Plus Blood Collection Tubes, Becton Dickinson) for subsequent analysis of βHB). A second blood sample was collected in plastic tubes (4 mL) containing sodium fluoride (Plus Blood Collection Tubes, Becton Dickinson). Samples were stored on ice before centrifugation at 3000*g* for 10 min at 4°C, after which three aliquots of plasma were separated for storage at −80°C until later analysis of plasma βHB, lactate, and glucose (RX Daytona, Randox Laboratories, UK: assay codes RB1007, LC2389, and GL364, respectively).

Statistical analysis. Data were evaluated using Prism 7.0 (GraphPad Software, Inc., CA) and are presented as mean (lower–upper 95% confidence interval [CI] of the mean), except the participant characteristics, which are described as mean ± SD. A paired samples *t*-test was used to determine differences between trials in run time to exhaustion and average HR during part B. Two-way (time–condition) repeated-measures ANOVA was used to determine differences between the two experimental trials for all other with variables with serial measurements. When a main effect of condition or an interaction effect between condition and time was indicated, *post hoc* testing was performed with Bonferroni's correction with multiplicity-adjusted *P* values applied to compare KE to PLA at the respective time points. The data were tested for normality using the Shapiro–Wilk test before proceeding with the parametric tests described. For null hypothesis statistical testing, the significance level was set at $\alpha = 0.05$ for all tests. Apart from and independent of the outcome of the repeated-measures ANOVA, standardized differences in the mean were used to assess magnitudes of effects between conditions at respective time points. These effect sizes were calculated using Cohen's *d* and interpreted using thresholds of <0.25, >0.25, >0.5, and >1.0 for trivial, small, moderate, and large, respectively (32).

RESULTS

Preexercise hydration status. Hydration status, measured as urine osmolality before each trial, did not differ between trials (mean [95% CI]: KE, 420 [259–581] mOsm·kg^{-1}; PLA, 460 [189–732] mOsm·kg^{-1}; $P = 0.645$, $d = 0.18$).

Plasma βHB, glucose, and lactate concentrations. Fasting plasma concentrations of βHB (mean [95% CI]: KE, 0.11 [0.09–0.13] mM; PLA, 0.11 [0.09–0.13] mM; $P > 0.99$), glucose (KE, 4.81 [4.62–5.00] mM; PLA, 4.84 [4.57–5.01] mM; $P > 0.99$), and lactate (KE, 0.86 [0.71–1.02] mM; PLA, 0.96 [0.82–1.11] mM; $P > 0.99$) concentrations did not differ between trials (Fig. 2). A main effect of time and condition (both $P < 0.001$) and a time–condition interaction effect ($P < 0.001$) were observed for plasma βHB concentrations (Fig. 2A). Ingestion of KE resulted in a rise in plasma βHB concentrations to 1.05 mM (95% CI = 0.83–1.26 mM) ($P < 0.001$) by the start of exercise. Concentrations continued to rise throughout exercise with the highest concentrations during KE observed at cessation of shuttle run to exhaustion at 2.61 mM (95% CI = 2.03–3.10 mM) ($P < 0.001$).

A main effect of time ($P < 0.001$) and condition ($P = 0.020$) was observed for plasma glucose concentrations (Fig. 2B). Plasma glucose concentrations were lower during KE compared with PLA at each time point except block 3, but *post hoc* pairwise comparisons did not reveal significant differences between conditions at any time point. However, standardized differences in the mean indicated moderate effect sizes at each of these time points. Specifically, plasma glucose concentrations were lower during KE compared with PLA by 10.9% (−0.56 [−1.48 to 0.35] mM; $d = 0.52$) after block 1, 7.5% (−0.48 [−1.40 to 0.44] mM; $d = 0.56$) after block 2, 11.6% (−0.76 mM [−1.68 to 0.15] mM; $d = 0.80$) after block 4, and 8.4% (−0.55 [−1.46 to 0.37] mM; $d = 0.56$) after block 5. There was no difference in plasma glucose concentration at the end of the shuttle run to exhaustion (KE, 6.49 [6.01–6.90] mM; PLA, 6.46 [5.64–7.23] mM; $P > 0.99$, $d = 0.04$).

A main effect of time and condition (both $P < 0.001$) and a time–condition interaction effect ($P = 0.009$) were

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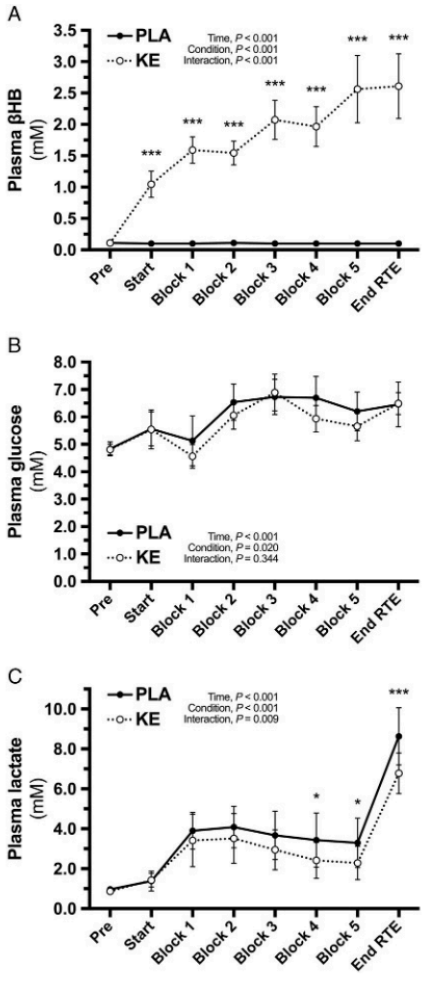

FIGURE 2—Plasma βHB (A), glucose (B), and lactate (C) concentrations during each trial. Data are presented as mean values, with error bars representing 95% CI. *$P < 0.05$ KE vs PLA; ***$P < 0.001$ KE vs PLA.

observed for plasma lactate concentrations (Fig. 2C). Plasma lactate concentrations were elevated from block 1 onward during both conditions but were lower during KE compared with PLA at each time point. *Post hoc* pairwise comparisons revealed significant differences between conditions at block 4 ($P = 0.037$), block 5 ($P = 0.042$), and the end of the shuttle run to exhaustion ($P < 0.001$), and effect sizes indicating small, moderate, and large effects across all time points, i.e., block 1: −11.9%, −0.48 mM (−1.46 to 0.50), $d = 0.27$; block 2: −13.8%, −0.56 mM (−1.54 to 0.42), $d = 0.33$; block 3: −20.2%, −0.73 mM (−1.71 to 0.25), $d = 0.45$; block 4: −29.3%, −1.02 mM (−2.00 to −0.04), $d = 0.58$; block 5: −30.1%, −1.00 mM (−1.98 to 0.02), $d = 0.63$; end of the shuttle run to exhaustion: −21.5%, −1.85 mM (−2.83 to −0.87), $d = 1.00$).

HR and RPE. For both HR and RPE, main effects of time were observed (both $P < 0.001$), but the absence of main effects of condition or time–condition interaction effects indicates that ingestion of KE did not alter the HR or RPE response during any block of part A of the LIST protocol (Fig. 3). However, the average HR during the shuttle run to exhaustion was lower (−3.9 [−6.4 to −1.4] bpm; $P = 0.007$, $d = 0.42$) during KE (170.7 [163.4–177.9] bpm) compared with PLA (174.6 [168.3–180.8] bpm).

Fifteen-meter sprint times and shuttle run time to exhaustion. A main effect of time was observed for 15-m sprint times during part A ($P < 0.001$), but no main effect of condition or time–condition interaction effect was observed (Fig. 4A). There was no statistically significant difference in the shuttle run time to exhaustion (KE, 229 [178–280] s; PLA, 267 [199–336] s; $P = 0.126$), but standardized differences in the mean indicated a small effect size for this difference (−38 [−89 to 13] s; $d = 0.45$).

Cognitive performance. A time–condition interaction effect ($P = 0.021$) was observed for the number of incorrect responses in the executive function multitasking test, which increased from pre- to postexercise in PLA (1.8 [−0.6 to 4.1]), but not in KE (0.0 [−1.8 to 1.8]) ($P = 0.017$, $d = 0.70$) (Table 1). The absence of main effects for time or condition and the time–condition interaction effects indicate that there

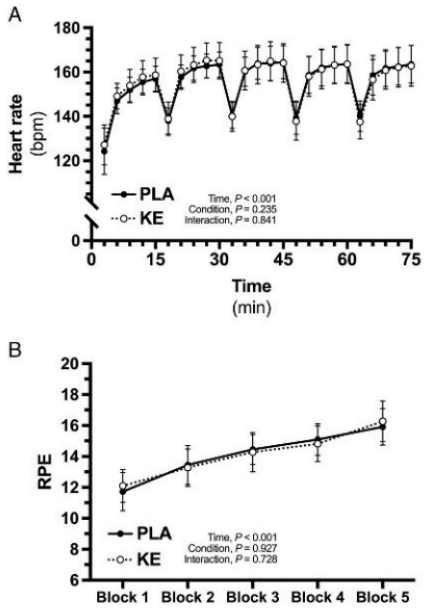

FIGURE 3—HR (A) and RPE (B) during each trial. Data are presented as mean values, with error bars representing 95% CI.

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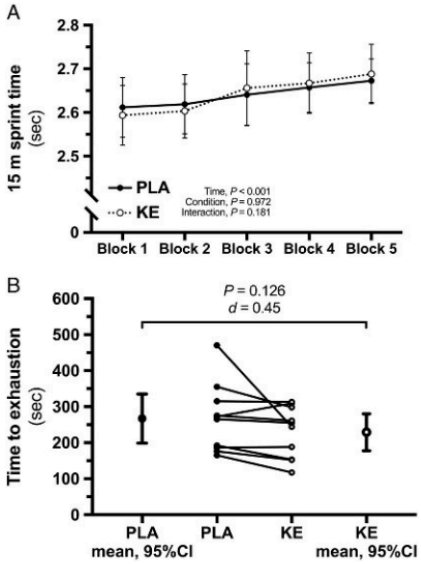

FIGURE 4—Fifteen-meter sprint times (A) and shuttle run time to exhaustion (B) during each trial. Data are presented as mean values, with error bars representing 95% CI.

was no difference between conditions in either reaction time or rapid visual information processing assessed by a sustained attention task (Table 1).

GI symptoms. Out of 11 participants, 4 (36%) reported symptoms of GI distress during PLA and comprised 4 (36%), 3 (27%), 3 (27%), 1 (9%), and 1 (9%) reports of belching, cramps, flatulence, boating, and nausea, respectively. Out of 11 participants, 9 (82%) reported symptoms of GI distress during KE. These comprised 7 (64%), 6 (55%), 4 (36%), 3 (27%), 3 (27%), and 1 (9%) of the participants reporting nausea, cramps, belching, heartburn, flatulence, and vomiting, respectively.

Identification of KE and best performance trials. Out of 11 participants, 8 (73%) correctly identified the trial in which they received KE, identifying KE by the awareness of taste and GI symptoms. However, only five (45%) of the participants correctly identified the trial in which they performed better in the part B run to exhaustion. Only three participants (27%) stated that they believed KE ingestion improved their performance, and two out of those three participants correctly identified their KE trial and their best performance.

DISCUSSION

The aim of the present study was to investigate the effect, if any, of the acute ingestion of a ketone ester on metabolic responses, physical performance, and cognitive performance in team sport athletes in response to an intermittent running

TABLE 1. Cognitive performance measures assessed before and after each trial.

	Reaction Time Test											
	Release Time (ms)			Reaction Time (ms)			Errors					
	Pre	Post	Post-Pre	Pre	Post	Post-Pre	Pre	Post	Post-Pre
KE	393 (360 to 425)	394 (369 to 419)	1 (16 to 18)	222 (181 to 263)	218 (177 to 259)	−4 (−39 to 31)	0.4 (−0.1 to 0.8)	0.8 (0.0 to 1.5)	0.4 (−0.6 to 1.4)
PLA	404 (371 to 436)	397 (368 to 427)	−6 (−31 to 19)	225 (172 to 278)	237 (205 to 269)	12 (−10 to 34)	0.8 (−0.2 to 1.7)	0.6 (−0.6 to 1.8)	−0.1 (−1.0 to 0.7)
d			−0.28			0.45			−0.46
	Multi-tasking Test											
	Response Latency (ms)*			Correct Responses**			Incorrect Responses**		
	Pre	Post	Post-Pre	Pre	Post	Post-Pre***	Pre	Post	Post-Pre***
KE	590 (510 to 669)	550 (483 to 616)	−40 (−78 to −2)	157.9 (156.4 to 159.3)	157.9 (156.0 to 159.7)	0.0 (−1.8 to 1.8)	2.1 (0.7 to 3.6)	2.1 (0.3 to 4.0)	0.0 (−1.8 to 1.8)
PLA	589 (526 to 652)	543 (499 to 587)	−46 (−74 to −19)	157.8 (155.7 to 159.8)	156.0 (152.8 to 159.2)****	−1.8 (−4.1 to 0.6)	2.3 (0.2 to 4.3)	4.0 (0.8 to 7.2)****	1.8 (−0.6 to 4.1)
d			−0.16			−0.70			0.70
	Rapid Visual Information Processing Test											
	Response Latency (ms)			Correct Responses			False Alarms		
	Pre	Post	Post-Pre	Pre	Post	Post-Pre	Pre	Post	Post-Pre
KE	449 (389 to 509)	430 (365 to 495)	−19 (−65 to 26)	44.0 (35.7 to 52.3)	44.6 (36.9 to 52.3)	0.6 (−2.7 to 4.0)	2.0 (0.9 to 3.1)	2.3 (0.5 to 4.0)	0.3 (−0.9 to 1.4)
PLA	460 (395 to 425)	446 (383 to 510)	−14 (−43 to 16)	45.3 (39.0 to 51.5)	44.8 (37.4 to 52.1)	−0.5 (−5.7 to 4.7)	2.4 (1.1 to 3.6)	2.5 (0.8 to 4.2)	0. (−1.2 to 1.4)
d			0.12			−0.21			−0.08

Data are presented as mean (95% CI), $n = 8$. Effect size calculated as Cohen's *d*.
*$P < 0.01$ for main effect of time.
**$P < 0.05$ for condition–time interaction effect.
***$P < 0.05$ for KE vs PLA.
****$P < 0.05$ for Post vs Pre.

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protocol that simulated soccer play. Compared with carbohydrate ingestion alone (PLA), ingestion of the ketone ester with carbohydrate (KE) resulted in an elevation in plasma βHB concentration to >1.5 mM after 15 min of exercise and reached ~2.6 mM by the end of exercise. Metabolic consequences included reductions in plasma glucose and lactate concentrations compared with PLA, but no differences in HR or RPE were observed between conditions. KE was without benefit to 15-m sprint times throughout the simulated protocol, or endurance capacity measured by shuttle run to exhaustion. However, cognitive performance in a multitasking executive function test was preserved with KE but declined during PLA.

Recent reports have investigated the effect of acute ingestion of exogenous ketone supplements on physical performance in endurance athletes (10,14,15). Ingestion of a βHB KME increased distance covered in a 30-min cycling time trial by ~2% (411 ± 162 m) (10), whereas, by contrast, ingestion of an acetoacetate KDE impaired performance in a 31.2-km cycling time trial by 2% ± 1% (58.2 s) (15). The latter effect was explained by a reduction in average power output by 3.7% and coincided with a high prevalence of GI distress. In addition, ingestion of a βHB salt formulation had no effect on average power output during a 4-min maximal performance cycling test (14). A key distinction between these studies is the form of exogenous ketone supplement ingested. Acute ingestion of KME produces plasma βHB concentrations of ~3.0 mM after 20 min, but the KDE and racemic ketone salts result in blood βHB concentrations only in the 0.3- to 0.6-mM range (14–16). Although this concentration range constitutes acute nutritional ketosis and is sufficient to affect the metabolic response to exercise (12,16), performance is unlikely to be affected unless circulating βHB concentrations exceed 1.0 mM (20). In the present study, the KE condition resulted in an elevation of plasma βHB concentrations to >1.5 mM after 15 min of exercise and reached ~2.6 mM by the end of exercise, which is broadly similar to previous work (9,10). Our participants ingested 750 $mg \cdot kg^{-1}$ body mass split across three boluses with 50% ingested 20 min before commencing exercise, and the remainder split into aliquots of 25% ingested at 30 and 60 min of exercise, respectively. This feeding strategy mimics the previous work with KME ingestion and exercise performance, which resulted in plasma βHB concentrations of ~2 mM 20 min after ingestion and ranged from ~2.0 to 3.0 mM throughout 90 min of exercise (10). By contrast, our participants only achieved these levels 65 min after ingestion of the initial bolus of KE, but these participants ingested KE in the postprandial state as opposed to the fasted state in the KME work. Ingestion of KME in the postprandial state can attenuate the C_{max} of blood βHB concentrations by 33% and the 4-h βHB AUC by 27% (9).

Accordingly, despite the similar changes in circulating βHB concentrations to the previous investigation of performance effects using the KME (10), the KE condition in the present study was without benefit to 15-m sprint times throughout the 75-min intermittent running protocol (LIST part A), or on shuttle run time to exhaustion (LIST part B). The metabolic consequences of KME ingestion were recapitulated herein, namely, lower plasma glucose and lactate concentrations during KE compared with PLA. A 10% reduction in plasma glucose was observed 35 min after ingestion of the initial KE bolus and was 8% to 12% lower (moderate effects) during blocks 1, 2, 4, and 5 of part A. The glucose-lowering effect of exogenous ketones is well documented whether ingested alone (9,10,12,16,33) or in combination with carbohydrate and/or protein (9,10,13,15,34). Although the insulinotropic action of ketone bodies is not always observed (5,6), it can occur under certain conditions (2), including when KME is ingested in the fasted state (9,10,33). However, when coingested with carbohydrate and/or protein, the glucose-lowering effect of exogenous ketones occurs despite similar circulating insulin concentrations in response to carbohydrate and/or protein alone at rest (34), during exercise (10), and during recovery from exercise (13). A βHB-mediated glucose-lowering effect is likely a result of an attenuation of hepatic gluconeogenesis and increase in hepatic glucose uptake (6). The rise in plasma lactate concentrations was attenuated during KE compared with carbohydrate ingestion alone, consistent with previous KME work (10). An attenuation in the exercise-induced rise in plasma lactate was previously explained by a reduction in glycolytic flux, sparing of muscle glycogen during exercise, and an increased contribution of ketone bodies and intramuscular triglycerides to energy provision (10). Whereas a 50% reduction in the rise in plasma lactate was observed during a 60-min preload at 75% W_{max} and 30 min time trial in trained cyclists (10), we observed a reduction ranging from ~10% to 30%. Given the lower aerobic fitness in our team sport athletes, the trained cyclists may have had a greater capacity to extract ketones from circulation and oxidize them as a substrate, resulting in a larger contribution toward total energy production and a greater reduction in glycolytic flux. This is because ketone bodies are transported across the skeletal muscle membrane by monocarboxylate transporters, which are most highly expressed in type I muscle fibers, and are increased in response to endurance exercise training (35).

For that reason, we previously hypothesized that performance benefits of exogenous ketones are most likely to be realized in those individuals with high levels of aerobic fitness and higher proportions of type I muscle fibers and/or monocarboxylate transporter expression (20). A lower level of aerobic fitness and training status, and therefore lesser ability to oxidize circulating ketones, may be one explanation for the lack of performance benefit in the present work. That notwithstanding, our performance test was shorter (~2 to 6 min) and intermittent in nature, which may be another factor contributing to the contrasting results. Another explanation may relate to the proposed benefits of exogenous ketone supplements being via their glycogen sparing effect. Given that we used an optimal carbohydrate-based fuelling on the

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day before and the day of each trial, our athletes may not have experienced glycogen depletion to an extent that the purported glycogen sparing would have benefited performance in part B. In fact, standardized differences in the mean used to assess magnitudes of effects between KE and PLA indicate a small effect size for a decrement in performance with KE, so it would be remiss not to consider that the effect of exogenous ketones in this instance may have been to impair carbohydrate utilization.

Nutrition strategies such as high fat feeding, ketogenic diets, and exogenous ketone ingestion alter substrate utilization during exercise, which generally results in lower rates of carbohydrate utilization at moderate-to-high exercise intensities (18–20). Whether this shift in substrate utilization reflects a sparing of muscle glycogen, which can then be used later in an exercise challenge, or instead reflects an impairment of muscle glycogen utilization during such exercise intensities is a salient issue for alternative fuelling strategies. The mechanistic basis for reduced carbohydrate utilization in the presence of exogenous ketones is proposed as an attenuation of glycolytic flux via inhibition of pyruvate dehydrogenase (PDH) and phosphofructokinase by increases in NADH:NAD^+, acetyl-CoA:CoA, or citrate. A similar mechanism is likely to contribute to the impaired performance during moderate- to high-intensity efforts observed under high fat feeding (36,37). The attenuation of PDH activity under such conditions (38) could be problematic for intermittent activity sports that require high-intensity efforts, which rely heavily on ATP provision from glycolytic pathways, performed on a moderate-intensity background. Clearly, this is the nature of the exercise challenge in the present study, but future work will require direct measurement of PDH activity and glycolytic flux in muscle biopsies to make definitive conclusions about the effects of exogenous ketones on utilization of muscle glycogen in this model. Conversely, we observed no benefit or decrement on 15-m sprint times performed at a rate of approximately nine sprints per 15-min block across 75 min of intermittent activity. Maximal short duration sprints rely primarily on the ATP–phosphocreatine system and anaerobic glycolysis for energy provision, but as the number of repeated sprints increases, the contribution of both energy systems decline and the contribution of aerobic glycolysis of circulating glucose and muscle glycogen increase over time (39,40). The lower plasma lactate concentrations in part A during KE suggests a reduction in glycolytic flux, but the reduction ultimately did not affect performance in repeated sprints of <3-s duration.

A higher incidence of GI symptoms occurred during KE compared with PLA, although this did not affect the HR or RPE responses during exercise. GI symptoms are a common side effect of KME and KDE ingestion, and more work is needed on the dose and timing of both these supplements to mitigate this response. KME ingested as part of a meal replacement milkshake drink causes a stepwise increase in symptoms with increasing dosages (7). Furthermore, ingestion of 500 $mg \cdot kg^{-1}$ body mass of KDE split in two doses caused symptoms in all participants during a cycling time trial (15). These symptoms are likely to be a large contributor to the performance decrement in that study given the participants' nomination of their symptoms as a distraction or interference to performance. The incidence of GI symptoms was higher in the present study than in previous work with KME (10), but the aforementioned commencement of exercise in a fed as opposed to fasted state, or this protocol involving running as opposed to cycling exercise, may be contributing factors.

A novel finding herein is the preservation of executive function during KE compared with PLA, measured by the number of incorrect responses to a multitasking test. Given that team sport athletes are presented with a multitude of decisions throughout match play, interventions that preserve or improve cognitive performance could positively influence performance outcomes. The primary physiological role of ketogenesis as a survival mechanism during low carbohydrate availability is providing a substrate to the brain in the presence of diminishing blood glucose concentrations (26). Cognitive benefits and a neuroprotective role are established for exogenous ketones in nonexercise contexts (27–29,41). Notably, in a short-term (5-d) feeding study, rats supplemented daily with KME were 38% faster at completing a radial maze task and made more correct decisions before making a mistake during the test (29). This outcome is consistent with our findings and suggests that central effects may be relevant during exercise, although other tests of cognitive function, i.e., reaction time and sustained attention tasks, were unaffected.

In conclusion, in team sport athletes, acute ingestion of a ketone ester elevated plasma βHB concentrations but did not improve performance in a shuttle run to exhaustion performed after 75 min of intermittent running. Reductions in plasma glucose and attenuated increases plasma lactate during exercise demonstrate the obvious effects of exogenous ketone ingestion on carbohydrate metabolism during exercise. However, participants experienced incidences of GI symptoms. These results underscore the need for future work to explore possible dose–response effects while minimizing any GI distress to athletes. Despite the lack of benefit to physical performance, the novel finding of preserved executive function after exhausting exercise suggests that there remains a possibility that exogenous ketones could enhance sport-specific performance of team sport athletes via other mechanisms.

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The authors declare that the results of the study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation and do not constitute endorsement by the American College of Sports Medicine.

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Appendix D: Accepted and published ahead of print Medicine and Science in Sport and Exercise article

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No Benefit of Ingestion of a Ketone Monoester Supplement on 10-km Running Performance

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ABSTRACT

Purpose: Pre-exercise ingestion of exogenous ketones alters the metabolic response to exercise, but effects on exercise performance have been equivocal. **Methods:** On two occasions in a double-blind, randomized crossover design, eight endurance-trained runners performed 1 h of submaximal exercise at ~65% VO_{2max} immediately followed by a 10-km self-paced TT on a motorized treadmill. An 8% carbohydrate-electrolyte solution was consumed before and during exercise, either alone (CHO+PLA), or with 573 $mg.kg^{-1}$ of a ketone monoester supplement (CHO+KME). Expired air, heart rate (HR), and rating of perceived exertion (RPE) were monitored during submaximal exercise. Serial venous blood samples were assayed for plasma glucose, lactate and β-hydroxybutyrate concentrations. **Results:** CHO+KME produced plasma β-hydroxybutyrate concentrations of ~1.0 to 1.3 mM during exercise ($P < 0.001$), but plasma glucose and lactate concentrations were similar during exercise in both trials. VO_2, running economy, respiratory exchange ratio, HR and RPE were also similar between trials. Performance in the 10-km TT was not different ($P = 0.483$) between CHO+KME (mean = 2402 s; 95% confidence interval [CI] = 2204, 2600 s) and CHO+PLA (mean = 2422 s; 95% CI = 2217, 2628 s). Cognitive performance, measured by reaction time and a multi-tasking test, did not differ between trials. **Conclusion:** Compared with carbohydrate alone, co-ingestion of KME by endurance-trained athletes elevated plasma β-hydroxybutyrate concentrations, but did not improve 10-km running TT or cognitive performance.

KEYWORDS: athletes; β-hydroxybutyrate; cognition; endurance; lactate; time trial;

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INTRODUCTION

The therapeutic and performance potential of exogenous ketone supplements has been the subject of increasing interest in recent years (1, 2). Metabolic effects the ketone bodies (KB), namely β-hydroxybutyrate (βHB) and acetoacetate (AcAc), are well-established in many organs, including attenuation of glycolysis, hepatic glucose output and adipose tissue lipolysis (3), but their potential role in modulating substrate utilization has garnered attention for athletic performance (4, 5). In the fasted state, KB provide up to 10% of energy to skeletal muscle during exercise (6), and after acute ingestion of exogenous ketone supplements, this contribution can apparently increase to 16 to 18% when circulating βHB is elevated to the 3 to 4 mM range (5). Moreover, this increase in βHB oxidation coincides with a reduction in glycolytic flux, as evidenced by an attenuation in the exercise-induced rise in plasma lactate and glycolytic intermediates, and an increase in intramuscular triglyceride utilization during exercise (5).

Circulating KB concentrations are <0.1 mM in the postprandial state, whereas hyperketonaemia is accepted as KB concentrations exceeding 0.2 mM (3). Ingestion of a variety of exogenous ketone supplements can acutely produce nutritional ketosis (4, 5, 7-17), which has been defined as circulating KB concentrations >0.5 mM (18). The most potent of these exogenous ketone supplements is the (R)-3-hydroxybutyl (R)-3-hydroxybutyrate ketone monoester (KME). When ingested at rest in the fasted state, KME produces a dose-dependent increase in circulating βHB concentrations of up to 6 mM 20 min after the ingestion of up to 573 mg kg^{-1} body mass (5, 9). This elevation in βHB concentration coincides with decreases in plasma glucose, free fatty acids (FFAs), triglycerides and ghrelin concentrations (5, 8, 9, 13). Exercise attenuates the rise in βHB concentrations, as ingestion of 573 $mg.kg^{-1}$ body mass KME prior to 45 min cycling at 45% and 75% peak power output (W_{max}) resulted in circulating βHB of ~4.0 mM and ~3.0 mM, respectively. As a consequence of the aforementioned effects on

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substrate utilization, acute ingestion of KME attenuates the rise in plasma glucose and lactate concentrations during exercise, whether in an endurance cycling or intermittent running context (4, 5).

These metabolic consequences have been proposed to explain the observation that the co-ingestion of KME in addition to a carbohydrate-based fuelling strategy improved performance in a 30 min maximum distance cycling time-trial by 2% when preceded by 1 h of submaximal 'pre-load' exercise (5). In contrast, high-intensity shuttle running capacity (~4 to 6 min) performed after 75 min of intermittent running was not improved in team sport athletes with KME co-ingestion compared to carbohydrate alone (4). While the former study considered a 'sparing' of muscle glycogen to be major factor in the performance benefit (5), the latter study speculated that the attenuation of glycolytic flux in the presence of elevated circulating βHB may have been a factor in the lack of performance benefit in that exercise model (4). Performance in exercise of long duration that incorporates high intensity efforts (i.e. sprint finishes, climbs) is largely dependent on carbohydrate utilization (19). Therefore, nutrition strategies that could spare muscle glycogen and maintain high intensities in the latter parts of races are of interest to scientists and practitioners (20). However, if glycogen sparing occurs via an attenuation of glycolytic flux that cannot be overcome when higher intensity efforts are required, this would instead be likely to impair performance (19). Moreover, the recent observation that acute ingestion of KME prior to intermittent exercise in team sport athletes resulted in preserved executive function as measured by a decision-making task after volitional exhaustion (4), remains to be confirmed in other exercise settings.

Therefore, the aim of the present study was to investigate the effects of acute ingestion of an exogenous ketone supplement in the form of a commercially-available KME on physiological

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responses, and physical and cognitive performance in endurance-trained runners in response to 1 h submaximal exercise immediately followed by a 10-km time trial.

METHODS

Participants. Eight trained, middle and long distance runners (M/F, 7/1; age, 33.5±7.3 y; height, 1.79±0.07 m; body mass, 68.8±9.7 kg; body fat, 8.0±4.1%; VO_{2max}, 62.0±5.6 mL.kg^{-1}.min^{-1}) gave written informed consent to participate after written and verbal explanations of the procedures. Ethical approval (permit number: DCUREC2018_039) was obtained from the Dublin City University Research Ethics Committee in accordance with the Declaration of Helsinki.

Experimental design. Participants visited the laboratory for exercise tests on four separate occasions over a 21 to 28 day period, comprising one baseline, one familiarization and two main experimental trials. During their first visit to the lab, each participant's maximal rate of oxygen consumption (VO_{2max}) was determined using an incremental treadmill test to volitional exhaustion. The exercise protocol performed in the familarization visit (visit 2) and two main experimental trials (visits 3 and 4) comprised of a pre-load of 1 h of treadmill running at 65%VO_{2max} followed by a self-paced 10-km time-trial (TT) performance test performed on a motorized treadmill (Fig. 1). A battery of cognitive tests was performed before and after the exercise protocol. The main experimental trials were performed in a double-blind, placebo-controlled, randomized crossover design. Visits 2, 3 and 4 were identical in terms of the pre-test preparation (standardized physical activity and diet for 24 h prior to each visit) and the exercise protocol. The visits differed only in the drinks consumed before and during exercise, namely an 8% carbohydrate-electrolyte solution, which was co-ingested with either a flavored placebo condition (CHO+PLA), or included the (R)-3-hydroxybutyl (R)-3-hydroxybutyrate ketone monoester (CHO+KME). The primary outcome was endurance performance measured by time to

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complete the self-paced 10-km TT, with secondary outcomes including cognitive performance, oxygen consumption (VO_2), running economy, respiratory exchange ratio (RER), heart rate (HR), rating of perceived exertion (RPE), and plasma βHB, glucose, and lactate concentrations.

Assessment of VO_{2max} and submaximal running speeds. Body mass was measured to the nearest 0.2 kg using a calibrated digital scales (SECA, Hamburg, Germany), and height was measured to the nearest 0.01 m using a wall-mounted stadiometer (Holtain, Crymych, UK). Body fat was determined by bioelectrical impedance analysis (DC-430U Dual Frequency Analyzer; Tanita, Arlington Heights, IL USA). All exercise testing and experimental trials were conducted on a motorized treadmill (T200; COSMED, Rome Italy). Initially, for the determination of the responses in VO_2 and blood lactate concentration at submaximal running speeds, participants ran for 4 min stages at a progressively increasing speeds, interspersed with a 1 min rest interval for determination of blood lactate concentrations (Lactate Pro 2; Arkray, Kyoto, Japan), RPE (Borg scale) and HR (Polar H7; Polar, Kempele Finland). The first stage was 4 $km.h^{-1}$ slower than the average speed corresponding to each participant's personal best time for a 10-km race. For each subsequent stage, the running speed was increased by 1 $km.h^{-1}$ until the running speed exceeded the speed corresponding to their personal best 10-km race speed. After a 10 min rest, participants began running at a speed corresponding to the last completed speed of the preceding test. Treadmill speed was increased by 2.0 $km.h^{-1}$ every 2 min for two stages, after which treadmill gradient was increased by 1.0% every 1 min until volitional fatigue. Expired air was collected and analyzed throughout these tests using the Quark RMR metabolic cart (COSMED, Rome, Italy). VO_2, carbon dioxide production (VCO_2), and RER were calculated from an average of breath-by-breath measurements during the last 30 s of each stage during the submaximal running stages and the assessment of VO_{2max}. VO_{2max} was considered to have been achieved if two of the following criteria were achieved: (i) plateauing of

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VO_2 despite increasing treadmill speed (increase in VO_2 of less than 2.0 $mL.kg^{-1}.min^{-1}$), (ii) HR within 5% of the age-predicted HR_{max} (208 – 0.7 x age in years), and (iii) an RER ≥1.10.

Cognitive test battery. The battery of cognitive tests (CANTAB Cognition, Cambridge, UK) was administered via a touch screen tablet lasting ~10 min. An identical test battery was administered before and after each trial in visits 2, 3 and 4.

During the reaction time (RTI) test, participants select and hold a button at the bottom of the screen and five circles are presented above. In each case, a yellow dot appears in one of the five circles, and the participants must react as soon as possible, releasing the button at the bottom of the screen, and selecting the circle in which the dot appeared. Release time (msec), reaction time (msec), and number of errors were recorded.

The multi-tasking test (MTT) is a test of executive function that measures the participant's ability to switch attention between stimuli, and ignore task-irrelevant information. White arrows are displayed on a black background, with the arrows located on either the left or right side of the screen, and pointing either to the left or to the right. A cue is displayed at the same time as the arrows, reading either "SIDE" or "DIRECTION". When the "SIDE" cue is presented, the participant is required to press a button on the left or right of the screen corresponding to the side of the screen where the arrow is presented, regardless of the direction the arrow is pointing. Conversely, when the "DIRECTION" cue is presented, the participants are required to touch a button on the left or right of the screen corresponding to the direction the arrow is pointing, regardless of which side of the screen the arrow is presented. Reaction time (msec), and number of correct and incorrect responses were recorded.

Pre-trial preparation. All experimental trials commenced between 0730 and 1130, and were completed within a period of 4.0-4.5 h (Fig. 1). On an individual basis, participants performed their second main experimental trial at the same time ±1 h as their first main trial. Pre-

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trial preparation was the same for the familiarization visit and each main experimental trial. Participants were asked to abstain from alcohol for 48 h and caffeine for 24 h, and refrain from strenuous exercise training on the day prior to each trial. For the day prior to experimental trials, participants were provided with a prescribed meal plan that provided ~2800 kcal (~41 kcal.kg^{-1}) at a macronutrient ratio of 60% carbohydrate (~6.2 g.kg^{-1}), 20% protein and 20% fat. Participants performed the two main experimental trials separated by either 7 or 14 days.

Main experimental trials. The protocol for the familiarization and main experimental trials were identical except for the drinks consumed before and during exercise (Fig. 1). Participants arrived to the laboratory in a fasted state 2 h prior to the commencement of exercise, and immediately consumed a standardized breakfast of quick-cook porridge oats and cereal bars providing ~300-400 kcal (~4.4-5.8 kcal.kg^{-1}) and ~1.0 g.kg^{-1} of carbohydrate, and 500 mL of water. Participants proceeded to complete the cognitive test battery 45 min after breakfast. Thereafter, an indwelling catheter (21G Insyte Autoguard; Becton Dickinson, Franklin Lakes, NJ USA) was introduced into an antecubital vein for serial blood sampling at rest (-30 and 0 min), during submaximal exercise (20, 40 and 60 min) and immediately after the 10-km TT.

For each trial, a bolus of a given drink was ingested 30 min prior to exercise (drink 1), at 20 min intervals during the 1 h of submaximal running (drinks 2 to 4), and at the 5-km mark of the 10-km TT (drink 5) (Fig. 1). The carbohydrate-based fuelling strategy (CHO) consisted of a 6.4% carbohydrate-electrolyte solution (Lucozade Sport; Lucozade Ribena Suntory Ltd., Uxbridge, UK) with maltodextrin (Cargill Inc, Minneapolis, MN USA) added to make an 8.0% carbohydrate-electrolyte solution that was provided at a rate of ~1.0 g.min^{-1} of exercise. During CHO+PLA, CHO was supplemented with denatonium benzoate, malic acid and arrow root extract to mimic the bitter taste and mouth-feel of the KME. During CHO+KME, CHO was supplemented with 573 mg.kg body mass^{-1} of a (R)-3-hydroxybutyl (R)-3-hydroxybutyrate

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ketone monoester (HVMN™ Ketone; HVMN, Inc., San Francisco, CA USA). The commercially-available ketone ester was mixed directly with the carbohydrate-electrolyte solution for ingestion, and the 573 $mg.kg^{-1}$ dose was divided into three boluses at a ratio 50:25:25 ingested at -30 min (drink 1), 20 min (drink 2) and 60 min (drink 4), respectively (Fig. 1). During CHO+PLA, drinks 1, 2 and 4 were flavored with the bitter additives to taste match with CHO+KME, and in both trials, drinks 3 and 5 were provided as the unadulterated 8% carbohydrate-electrolyte solution. All drinks were administered in opaque drinks bottles.

For the exercise protocol, participants first performed a standardized 5 min warm up on the motorized treadmill (8 $km.h^{-1}$) followed by self-selected stretching. Participants then performed 1 h of treadmill running at a speed corresponding to ~65%VO_{2max} (Table 1). Immediately after completion of the 1 h pre-load, participants completed a 10-km TT. The pre-load followed by TT protocol was modeled on the previous work demonstrating a benefit of KME on cycling TT performance (5), and has been similarly applied to treadmill running in previous studies (21, 22). Prior to each TT, participants were told to complete the distance as fast as possible i.e. to race the 10-km. They were allowed to adjust the treadmill speed as often and by as much as desired by manually-adjusting a side-mounted control panel on the treadmill. Increments or decrements in speed were 0.1 $km.h^{-1}$ in response to each press of an up or down arrow button, respectively. The 10-km TT began with the participant accelerating from a standing start. Participants were blinded to the speed of the treadmill and the time elapsed at all times, but were aware of the distance covered throughout the TT, including the 5-km mark when drink 5 was provided. After completing the 10-km TT, participants completed the same cognitive test battery as completed prior to exercise.

Venous blood samples were collected at 30 min prior to exercise, at 20 min intervals during submaximal exercise, and immediately after the 10-km TT. HR and RPE were recorded at

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20 min intervals during submaximal exercise. Expired air was collected during the first 10 min, 25 to 30 min, and 55 to 60 min of the submaximal exercise for the monitoring of exercise intensity, and calculation of RER and running economy. Running economy is expressed as the volume of oxygen required to run 1 km relative to body mass ($mL.kg^{-1}.km^{-1}$) (23). Incidences of gastrointestinal (GI) symptoms were recorded by interview after each trial. At the end of visit 4, participants completed an exit interview in which they were asked whether they could identify the CHO+KME condition, and to identify which experimental trial they believed that they performed their best TT.

Blood analysis. Blood was collected in plastic tubes (2 mL) containing sodium heparin (Plus Blood Collection Tubes; Becton Dickinson, Franklin Lakes, NJ USA) for subsequent analysis of βHB. A second blood sample was collected in plastic tubes (4 mL) containing sodium fluoride (Plus Blood Collection Tubes; Becton Dickinson, Franklin Lakes, NJ USA) for subsequent analysis of glucose and lactate. All collection tubes were pre-chilled, and blood samples were stored on ice before centrifugation at 3000 *g* for 10 min at 4ºC, after which aliquots of plasma were separated for storage at -80ºC until later analysis. Plasma βHB was determined by colormetric assay as per the manufacturer's instructions (MAK041; Sigma-Aldrich, Arklow, Ireland). Plasma glucose and lactate were measured using the RX Daytona™ chemical autoanalyser and appropriate reagents as per the manufacturer's instructions (Randox Laboratories, Crumlin, UK: assay codes GL3815 and LC3980, respectively).

Statistical analysis. The required sample size was calculated *a priori* using performance in the 10-km TT as the primary outcome measure. Based on the reliability data for the pre-loaded 10-km TT protocol employed (22), the assessment of other running TT protocols (24), and the variability of real-world performance in races of similar distance (25), we estimated a coefficient of variation (CV) of 1.5% for performance in the 10-km TT. We aimed to detect a 2.5% change

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in 10-km TT performance based on the smallest worthwhile difference (SWD) described by Russell et al. for this pre-loaded 10-km TT protocol being 2.1% (22). Consequently, the sample size calculation at an α level of 0.05 and power (1-β) of 0.8 revealed that six participants would be sufficient to detect a 2.5% change in 10-km TT performance. However, considering the adequacy of sample sizes in similar studies, as a conservative measure we recruited a final sample size of n=8. Data were evaluated using Prism v8.0 (GraphPad Software, Inc., San Diego, CA USA) and are presented as mean [lower, upper 95% confidence interval (CI) of the mean], except for the participant characteristics, which are described as mean±SD. A one-way repeated measures analysis of variance (ANOVA) was used to determine whether a trial order effect existed across visits 2, 3 and 4 in the time to complete the 10-km TT. A paired samples t-test was used to determine differences between trials in time to complete the 10-km TT. The SWD was set at 0.2 between-subject SD, which is suggested to represent a practically-relevant change in performance in athletes. Thus, the SWD corresponded to 48 sec, or 2.0%, for 10-km TT performance in this study. Two-way (time x condition) repeated measures ANOVA was used to determine differences between the two experimental trials for all variables with serial measurements. When a main effect of condition, or an interaction effect between condition and time was indicated, *post-hoc* testing was performed with Bonferroni's correction with multiplicity-adjusted *P* values applied to compare CHO+KME to CHO+PLA at the respective time points. The data were tested for normality using Shapiro-Wilk test prior to proceeding with the parametric tests described. For null hypothesis statistical testing, the significance level was set at $\alpha = 0.05$ for all tests.

RESULTS

Plasma βHB, glucose and lactate concentrations. Postprandial plasma concentrations of βHB (mean [95% CI]: CHO+KME, 0.27 [0.22-0.33] mM; CHO+PLA, 0.28 [0.14-0.43] mM),

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glucose (CHO+KME, 3.96 [3.22-4.70] mM; CHO+PLA, 3.70 [3.06-4.35] mM), and lactate (CHO+KME, 1.04 [0.79-1.29] mM; CHO+PLA, 1.02 [0.84-1.20] mM) did not differ between trials (all $P > 0.99$). A main effect of time and condition (both $P < 0.001$) and a time-condition interaction effect ($P < 0.001$) were observed for plasma βHB concentrations (Fig. 2A). Ingestion of CHO+KME resulted in a rise in plasma βHB concentrations to 0.99 (0.85-1.14) mM at 0 min. βHB concentrations peaked at 1.33 (1.13-1.52) mM during submaximal exercise at 40 min, with similar concentrations observed at the cessation of the 10-km TT at 1.33 (0.95-1.70) mM.

A main effect of time ($P < 0.001$) and condition ($P = 0.027$) was observed for plasma glucose concentrations (Fig. 2B). Plasma glucose concentrations were lower in CHO+KME at 0 min, i.e. 30 min after ingestion of the first bolus of either CHO+KME or CHO+PLA (CHO+KME, 3.87 [3.22-4.70] mM; CHO+PLA, 4.52 [3.91-5.13] mM; $P = 0.016$) (Fig. 2B). Plasma glucose concentrations rose throughout submaximal exercise (Fig. 2B) with the highest concentrations observed at cessation of the 10-km TT (CHO+KME, 6.94 [5.60-8.28] mM; CHO+PLA 7.24 [5.93-8.54] mM), with no difference between trials ($P > 0.99$).

A main effect of time ($P < 0.001$) was observed for plasma lactate concentrations, but were similar between trials at all time points (Fig. 2C). Peak plasma lactate concentrations were observed at cessation of the 10-km TT (CHO+KME, 6.94 [4.15, 9.73] mM; CHO+PLA, 7.48 [5.46-9.51] mM; $P = 0.738$).

Submaximal exercise. Running speeds were identical between trials as per the study design. There was no difference in $\%VO_{2max}$, VO_2, running economy, VCO_2, RER, HR, and RPE between CHO+KME and CHO+PLA during the submaximal exercise period (Table 1). Main effects of time were observed for the decline in RER ($P < 0.001$), and the increase in RPE ($P < 0.001$) during the submaximal exercise bout (Table 1)

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10-km TT performance. No trial order effect was observed for 10-km TT performance between visit 2 (2388 [2187-2588] s), visit 3 (2415 [2223-2607] s), and visit 4 (2409 [2197-2621] s) ($P = 0.742$). There was no statistically significant difference (-20 [-86-45] s; $P = 0.483$) in 10-km TT performance between trials (CHO+KME, 2402 [2204-2600] s; CHO+PLA, 2422 [2217-2628] s) (Fig. 3A). Compared to CHO+PLA, three participants demonstrated improvements in performance with CHO+KME that were greater than the SWD, and one participant demonstrated a decrement in performance with CHO+KME that was greater than the SWD (Fig. 3B). The remaining participants' differences in performance between trials were less than the SWD. Running speeds for each 2 km split during the 10-km TT did not differ between trials, but did increase progressively throughout the TT (main effect of time, $P < 0.001$) (Fig. 3C).

Cognitive performance. In the reaction time test (RTI), main effects of time ($P = 0.026$) and condition ($P = 0.026$) were observed for release time, but no interaction effect was present ($P = 0.535$), whereas an interaction effect was observed for reaction time ($P = 0.014$) (Table 2). In the multi-tasking test (MTT), a main effect of time was observed for response latency ($P = 0.010$), correct responses ($P = 0.049$) and incorrect responses ($P = 0.036$), but no main effects of time, or interaction effects were observed across these parameters (all $P > 0.05$) (Table 2). Overall, there was no difference in cognitive performance between conditions in either the RTI, or MTT assessments (Table 2).

Gastrointestinal symptoms. Out of 8 participants, 4 (50%) reported symptoms of GI distress during CHO+PLA and comprised 4 (50%), 3 (38%), 1 (13%), 1 (13%), and 1 (13%) incidences of belching, flatulence, reflux, urge to defecate, and diarrhea, respectively. Out of 8 participants, 5 (63%) reported symptoms of GI distress during CHO+KME and comprised 3 (38%), 2 (25%), 1 (13%), 1 (13%), 1 (13%), and 1 (13%) incidences of belching, urge to defecate, cramps, reflux, nausea, and stitch, respectively.

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Identification of CHO+KME and best performance trials. Out of 8 participants, 2 (25%) correctly identified the trial in which they received CHO+KME, identifying CHO+KME by taste and a perceived alteration of performance. Six (75%) participants declared that they could not differentiate between CHO+PLA and CHO+KME. Seven (88%) participants correctly identified the trial in which they performed their best 10-km TT.

DISCUSSION

The present study investigated whether the acute ingestion of a commercially-available ketone monoester supplement altered metabolic responses, physical and cognitive performance in endurance-trained runners in response to 1 h of submaximal exercise immediately followed by a treadmill-based self-paced 10-km TT. Compared with placebo (CHO+PLA), ingestion of the ketone monoester (CHO+KME) elevated plasma βHB to ~1.0 mM at the onset of submaximal exercise, and reached ~1.3 mM at the end of the 10-km TT. However, CHO+KME did not alter the metabolic or cardiorespiratory responses to exercise, or demonstrate benefit to physical or cognitive performance compared to CHO+PLA ingestion.

The present study adds to the growing body of literature investigating the effects on exercise performance of elevating ketone body concentrations by exogenous means. The term "exogenous ketone supplement" encompasses a range of different forms of supplements, with each having differential effects on the metabolic response to exercise, and exercise performance. These studies have included the acute ingestion of a (R)-3-hydroxybutyl (R)-3-hydroxybutyrate ketone monoester (KME) (4, 5), and a R,S-1,3-butanediol acetoacetate ketone diester (KDE) (12), racemic ketone salts (KS) (7, 10, 11, 14), and the ketogenic compound 1,3-butanediol (BD) (21, 26) prior to and/or during an exercise challenge. One of the key metabolic consequences of ingesting exogenous ketone supplements is the elevation in circulating βHB, but we speculate that exercise performance is unlikely to be affected unless βHB concentrations exceed 1.0 mM

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(27). To date, the only supplement to consistently exceed this threshold prior to an exercise challenge is the KME supplement (4, 5). KS and KDE elevate βHB concentrations into the 0.3 to 0.6 mM range (7, 10, 12), and ingestion of BD elevates βHB concentrations into the 0.6 to 0.8 mM range (21, 26).

Specifically focusing on KME ingestion and exercise studies, ingestion of 573 $mg.kg^{-1}$ of KME in the fasted state elevated βHB concentrations to ~2.0 mM 20 min after ingestion where it remained throughout 1 h cycling exercise at 75%W_{max} and a subsequent 30 min TT (5). In the fed state, ingestion of 750 $mg.kg^{-1}$ of KME elevated βHB concentrations to >1.5 mM after 15 min of exercise, and ~2.6 mM by the end of 75 min of intermittent running followed by a short duration shuttle run to exhaustion (4). In contrast to this previous work, plasma βHB concentrations in the present study were elevated to ~1.3 mM during the exercise protocol, which is lower than previously observed at the same 573 $mg.kg^{-1}$ dose (5). These previous studies have used a split dosing strategy to achieve to the total KME dose described (4, 5), and therefore we employed the same approach. The presently-observed attenuated rise in plasma βHB concentrations compared to these studies is unsurprising given that ingestion of KME in the fasted states consistently elevates circulating βHB to >3.0 mM (8, 9), whereas ingestion of KME in the postprandial state results in circulating βHB in the range from ~1.0 to 2.5 mM (4, 5, 9). For instance, ingestion of 395 $mg.kg^{-1}$ in the fasted state produces peak βHB concentrations of ~3.0 mM but only ~2.0 mM in the fed state, a 33% reduction in C_{max} and coincides with a 27% reduction in 4 h βHB AUC in resting participants (9). Given that our participants were fed a lower initial dose of KME of 287 $mg.kg^{-1}$, that this ingestion occurred in a postprandial state, and that exercise commenced 30 min later, it is not surprising that we observed lower βHB concentrations prior to and during exercise compared to previous work (4, 5).

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Therefore, although the present protocol achieved acute nutritional ketosis, a benefit to endurance performance was not observed. This finding is consistent with a number of studies that have failed to find a performance benefit of exogenous ketone supplements in various exercise models (4, 10-12, 14). The variety of exogenous ketones supplements used, the large range of changes in circulating βHB produced, and a lack of consistency in the nutrients co-ingested and type of exercise challenge performed, make it difficult to make broad conclusions on the efficacy of these supplements. However, only one study to date has demonstrated a performance benefit with the ingestion of KME, which when co-ingested with CHO increased the distance covered in a 30 min cycling TT by ~2% (mean±SEM, 411±162 m; *n*=8), when preceded by 1 h pre-load exercise at 75% W_{max} (5). The proposed mechanism for this improvement in performance was a shift in the contribution to energy provision from substrate utilization of carbohydrate to fat, as demonstrated by reduction in glycolytic flux resulting in a 'sparing' of muscle glycogen, and a concomitant increase in intramuscular triglyceride utilization during exercise (5).

The mechanistic basis whereby elevated ketones reduce carbohydrate utilization during exercise is likely an attenuation of glycolytic flux via an inhibition of pyruvate dehydrogenase and phosphofructokinase by increases in NADH:NAD^+, acetyl-CoA:CoA, or citrate. A reduction in glycolytic flux has been proposed to explain the attenuated exercise-induced rise in plasma lactate observed in previous studies providing KME (4, 5). This attenuation was ~50% during 60 min at 75%W_{max} and 30 min TT in trained cyclists (5), and ~10% to 30% during 75 min of intermittent running in team sport athletes (4). However, no differences in plasma lactate were observed between trials in the present study either during the pre-load or TT periods. The submaximal exercise intensity of ~65%VO_{2max} employed was below lactate threshold for all participants, and therefore an intensity too low to observe an attenuation, if any, of the exercise-

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induced rise in plasma lactate. However, plasma βHB concentrations were elevated >1.0 mM before and at the cessation of the 10-km TT, yet no difference in plasma lactate was observed between trials.

Similarly, while a glucose-lowering effect of KME ingestion is well-documented whether ingested alone (5, 8, 9), or co-ingested with carbohydrate or protein (4, 5, 9, 13, 15), we observed an attenuation in the rise in plasma glucose concentrations only at 30 min after ingestion of the first bolus of CHO+KME compared to CHO+PLA. This difference in plasma glucose between trials was absent during the submaximal exercise period, and upon completion of the 10-km TT. When effects of KME ingestion on plasma glucose have been observed, the mechanism proposed has been an attenuation of hepatic gluconeogenesis and an increase in hepatic glucose uptake (13). Under certain conditions, elevated KB concentrations may have an insulinotropic action (6), but is not always observed (28, 29). When co-ingested with carbohydrate and/or protein, the effect of exogenous ketones to attenuate postprandial glycemia occurs despite similar circulating insulin concentrations between conditions (5, 13, 15).

We propose that the lack of differences between trials for plasma glucose and lactate, in contrast to previous work (4, 5), suggests that the nature of the exercise challenge, or the degree of nutritional ketosis are key determinants of the metabolic effects of exogenous ketone supplements during exercise. While plasma βHB concentrations were elevated to ~1.3 mM at the cessation of the 10-km TT, concentrations were ~1.2 mM lower than observed in studies demonstrating effects on plasma glucose and lactate during exercise (4, 5), The lower plasma βHB concentrations are a consequence of the aforementioned particulars of the dosing and feeding strategy, and future research should be cognizant of these issues when designing study protocols.

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The brain is the primary site of ketone body utilization under conditions of low carbohydrate availability (30). Elevated βHB concentrations are associated with a neuroprotective role in non-exercise contexts (31-33), and short-term (5 days) feeding of a diet supplemented with KME improved performance of rats in a radial maze task by 38%, and improved decision-making during the test (34). Moreover, in our previous work, acute ingestion of KME preserved cognitive performance, measured by the number of incorrect responses to a multi-tasking test (4). This test was performed at the cessation of a short duration intermittent run to exhaustion proceeding the Loughborough Intermittent Shuttle Test (LIST), a variable intensity running protocol that mimics soccer match-play (35). In contrast to previous results, we observed no difference in cognitive performance with the addition of KME in the present study. The specifics of the exercise challenge may play a role in these divergent findings. The LIST is a cognitively-demanding task that requires participants to be aware of current and subsequent running speeds for 75 min. Mental fatigue has a negative impact on aspects of cognitive performance, including altered attentional focus (36), and slower and less accurate reaction times (37), suggesting that the more cognitively-demanding the task, the larger a deficit in cognitive performance should be evident. In the present study, we observed no decline in cognitive performance in either condition. The absence of decline is important to note because in our previous work, it was a preservation of cognitive performance observed with KME, not an absolute improvement (4). These results suggest the exercise challenge presently employed was not sufficiently cognitively-demanding to negatively impact reaction time or executive function, and therefore, potential benefits were unlikely to be observed.

Concerns have been raised about the practical use of exogenous ketone supplements by athletes due to the high rates of occurrence of GI distress in previous work using BD (26), KS (7, 17), KDE (12), and KME (4). However, in the present study, incidences of GI distress were

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similar between conditions, and this is consistent with previous work using KME (5). Typically, rates of occurrence of GI distress are higher with exogenous ketones than with ingestion of water or carbohydrate alone, and GI distress occurs at a higher rate with increasing doses of exogenous ketones (4, 7, 38). Importantly, no participants nominated GI distress as a distraction or detriment to performance during CHO+KME trials.

The present study has attempted to incorporate several elements of experimental design that are consistent with reviews of best practice when undertaking studies of nutrition supplements and sports performance (24, 39-41). These include recruitment of trained participants who compete in the chosen mode of exercise, the inclusion of a familiarization trial to improve the reliability of the performance TT, standardization of nutrient intakes before and during each trial, the inclusion of an appropriate placebo coupled to the interrogation of the success of the blinding, and a fuelling strategy that mimics real-world practice i.e. exercise undertaken in fed conditions rather than fasted, and supported by optimal carbohydrate provision during performance. However, the study is not without limitations. While the sample size calculations suggested a small sample would be sufficient to detect meaningful change using this experimental protocol, an *n*-size of eight participants is underpowered to explore relationships between performance differences and inter-individual differences in VO_{2max} and peak plasma βHB concentrations achieved. These are two parameters that we speculate are important determinants of the performance benefits, if any, of exogenous ketone supplements (27). For example, that three out of eight participants had an improvement in 10-km TT performance that was greater than the SWD is suggestive of potential benefits to performance in certain athletes. Another limitation, despite the strength of the experimental protocol as described above, is that the performance measure employed lacks ecological validity, and is not an entirely accurate representation of a real-world performance scenario because of the pre-load protocol, and use of

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a motorized treadmill with self-paced adjustments of speed. The requirement for participants to manually change the treadmill speed using console buttons is dependent upon their perception of an ability to run faster or slower, but may not be sufficiently sensitive to detect small differences in performance (42, 43).

In conclusion, the addition of a commercially-available ketone monoester supplement to a carbohydrate-based fuelling strategy prior to and during exercise did not improve performance in a self-paced, treadmill-based 10-km TT. Ingestion of the ketone monoester attenuated the rise in plasma glucose prior to exercise, but concentrations were similar between trials thereafter, and no effect on the increase in plasma lactate concentrations during the 10-km TT was observed. Moreover, no differences between trials were observed for a range of physiological responses, and assessments of cognitive performance. Future research should evaluate different dosing strategies and exercise models to elucidate whether a threshold of plasma βHB concentration must be exceeded in order to exert performance benefits, and in which exercise contexts these benefits, if any, might be realized.

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The authors declare the results of the study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation and do not constitute endorsement by the American College of Sports Medicine.

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FIGURE LEGENDS

FIGURE 1 – Schematic of the study protocol. CHO, carbohydrate-electrolyte solution; HR, heart rate; KME, ketone monoester; PLA, placebo; RPE, rating of perceived exertion.

FIGURE 2 – Plasma βHB (A), glucose (B), and lactate (C) concentrations during each trial. Data are presented as mean values, with error bars representing 95% confidence intervals. *$P < 0.05$ for CHO+KME vs. CHO+PLA; ***$P < 0.001$ for CHO+KME vs. CHO+PLA.

FIGURE 3 – 10-km time-trial performance (A), individual differences between CHO+KME compared to CHO+PLA (B), and running speeds for each 2 km split during the 10-km time-trials (C). Data in (A) and (C) are presented as mean values, with error bars representing 95% confidence intervals. The shaded area in (B) represents the range for the smallest worthwhile difference in 10-km time-trial performance in this cohort.

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Figure 1

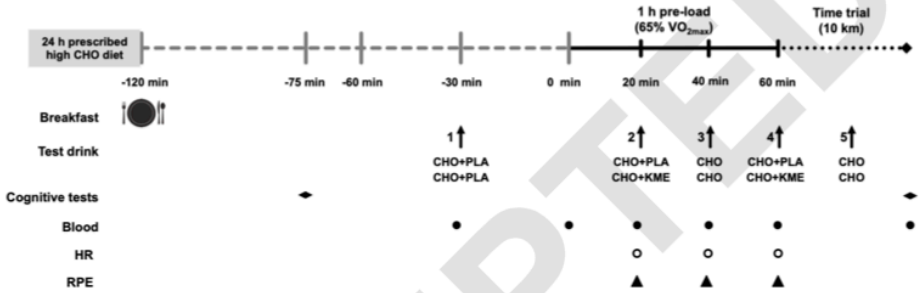

Figure 1

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Figure 2

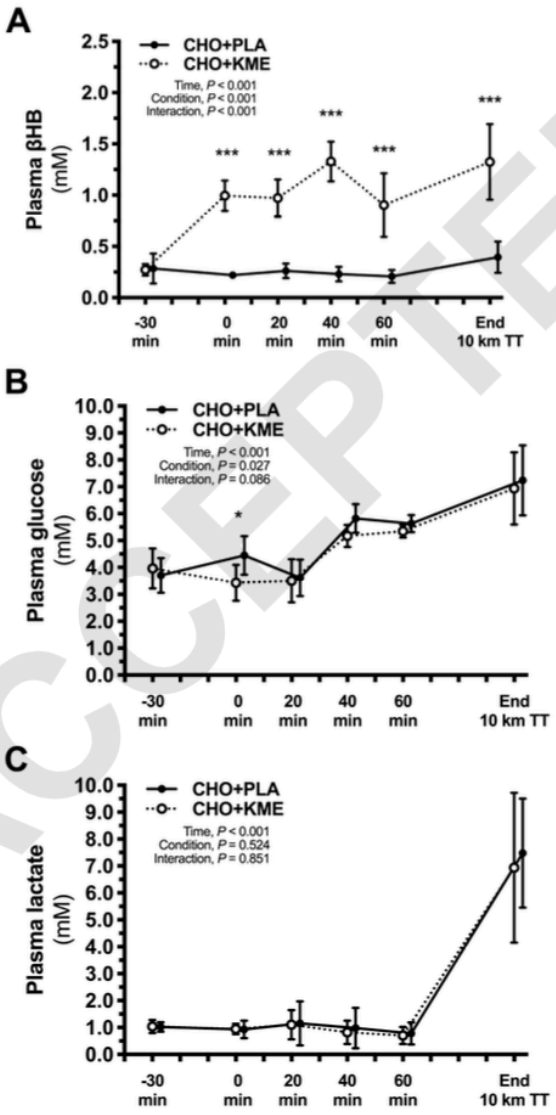

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Figure 3

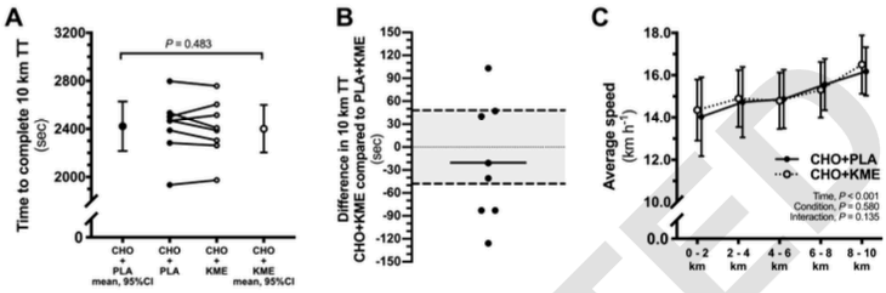

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TABLE 1. Physiological responses to 1 h of treadmill running at ~65%VO_{2max} when carbohydrate was co placebo (CHO+PLA) or a ketone monoester (CHO+KME).

	Time			
	0-10 min	**10-30 min**	**30-60 min**	
Running speed ($km.h^{-1}$)	12.4 (11.3, 13.5)	12.4 (11.3, 13.5)	12.3 (11.1, 13.5)	
VO_2 ($L.min^{-1}$)				Time,
CHO+PLA	2.84 (2.52, 3.16)	2.84 (2.56, 3.12)	2.81 (2.53, 3.09)	Condition,
CHO+KME	2.78 (2.42, 3.13)	2.79 (2.42, 3.13)	2.72 (2.49, 2.95)	Interaction,
%VO_{2max}				Time,
CHO+PLA	67.0 (62.8, 71.2)	66.9 (64.5, 69.4)	66.2 (63.8, 69.4)	Condition,
CHO+KME	65.3 (60.9, 69.8)	65.8 (62.6, 69.8)	64.1 (63.2, 65.0)	Interaction,
Running economy ($mL.kg^{-1}.km^{-1}$)				Time,
CHO+PLA	202 (184, 219)	203 (185, 220)	202 (185, 219)	Condition,
CHO+KME	196 (181, 212)	199 (181, 217)	196 (179, 213)	Interaction,
VCO_2 ($L.min^{-1}$)				Time,
CHO+PLA	2.67 (2.36, 2.99)	2.60 (2.30, 2.90)	2.55 (2.26, 2.84)	Condition,
CHO+KME	2.63 (2.28, 2.98)	2.58 (2.28, 2.89)	2.50 (2.26, 2.74)	Interaction,
RER				Time,
CHO+PLA	0.94 (0.92, 0.96)	0.91 (0.89, 0.94)	0.91 (0.88, 0.93)	Condition,

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CHO+KME	0.95 (0.92, 0.97)	0.92 (0.89, 0.96)	0.92 (0.89, 0.95)	Interaction,
HR (bpm)				Time,
CHO+PLA	141 (133, 149)	146 (137, 155)	145 (137, 154)	Condition,
CHO+KME	140 (131, 150)	144 (134, 154)	143 (134, 152)	Interaction,
RPE				Time,
CHO+PLA	10 (9, 12)	11 (10, 13)	12 (10, 13)	Condition,
CHO+KME	10 (8, 12)	11 (9, 12)	11 (9, 13)	Interaction,

Data are presented as mean (95% CI), n = 8. ***$P < 0.001$.

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TABLE 2. Measures of cognitive performance assessed before and after each trial consisting of 1 h of treadmill running at ~65% VO_{2max} followed by a 10-km time-trial during which carbohydrate was co-ingested with either placebo ketone monoester (CHO+KME).

	Reaction time test (RTI)						
	Release time[#,§] (msec)			Reaction time[†] (msec)			
	Pre	Post	Post-Pre	Pre	Post	Post-Pre	Pre
CHO+PLA	417 (373, 461)	401 (356, 446)	-16 (-35, 2)	223 (171, 276)	221 (165, 278)	-2 (-18, 14)	0.3 (-0.1, 0.6)
CHO+KME	430 (383, 477)	409 (368, 450)[*]	-21 (-40, -2)	214 (176, 252)	232 (183, 282)[*]	18 (2, 34)	0.6 (-0.3, 1.5)

	Multi-tasking test (MTT)						
	Response latency[#] (msec)			Correct responses[#]			
	Pre	Post	Post-Pre	Pre	Post	Post-Pre	Pre
CHO+PLA	599 (500, 698)	561 (447, 674)[***]	-38 (-58, -18)	159 (157, 160)	157 (155, 159)	-2 (-4, 1)	1 (0, 3)
CHO+KME	583 (513, 653)	541 (461, 622)[***]	-41 (-62, -21)	158 (157, 160)	157 (156, 159)	-1 (-4, 2)	2 (0, 3)

Data are presented as mean (95%CI), n=8. Symbols are $^{\#}P < 0.05$ for main effect of Time; $^{\S}P < 0.05$ for main effect of Condition; 0.05 for Time x Condition interaction effect; $^{*}P < 0.05$ for Post vs. Pre; $^{***}P < 0.001$ for Post vs. Pre.

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