Examining the Effects of Exogenous Ketones on Exercise Metabolism and Performance in Male Varsity Athletes

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final reversions, as accepted by my examiners.

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ABSTRACT

Previous research has shown that administration of exogenous acetoacetate (AcAc), beta-hydroxybutyrate (BHB) and glucose in a perfused rodent heart model can increase cardiac output while reducing oxygen uptake compared with glucose alone. With this apparent improvement in mechanical efficiency induced by AcAc and BHB, there is speculation that they may also enhance exercise performance in athletes. Although AcAc and BHB can be produced endogenously, exogenous BHB supplements have recently become commercially available in the form of ketone mineral salts (KS). Research to date has not found any performance enhancing effects of an acute dose of KS but the effects of more regular KS supplementation on whole body metabolism and exercise performance are unknown. Furthermore, medium-chain triglycerides (MCT) have recently been added to KS supplements, as the administration of KS plus MCT in rodents has been shown to increase blood BHB concentrations compared to KS administration alone. Therefore, the overall purpose of this study was to examine the hypotheses that a twice-daily KS plus MCT supplementation protocol would increase mechanical efficiency and improve exercise performance during a submaximal time to exhaustion (TTE) cycling protocol and a time trial (TT) cycling protocol in male varsity athletes. A single-blinded placebo-controlled crossover design was employed where participants were randomized to either a KS plus MCT supplement (0.3 g BHB/kg, 0.18 g MCT/kg 0.02 g Calcium/kg, 0.02 g Sodium/kg and 0.02 g Potassium/kg) or placebo (0.02 g Calcium/kg, 0.02 g Sodium/kg and 0.02 g Potassium/kg). Each phase of the study lasted 8 days. Seven male varsity athletes (25.6 ± 0.3 years of age, 87.7 ± 3.4 kg and 52.9 ± 3.1 mL/kg/min VO_2max) were recruited for this study. Prior to initiating the first phase, participants completed a cycling VO_2max test and two familiarization sessions to get accustomed to the TTE and TT cycling tests. On day 1 and 5 of each study phase, participants

reported to the laboratory for blood collection to assess whole blood [D-BHB] time course responses. On day 6, participants completed a TTE cycling protocol, consisting of a 5 minute warm-up at 50 Watts, followed by 20 mins at 50% of VO_2max. Following this work stage, a constant work load corresponding to 70% of VO_2max was maintained until participants reached fatigue. Forty-eight hours later (day 8), participants performed a 10-km cycling TT protocol where participants were instructed to complete the 10-km distance in the fastest time possible. Following the TT test, there was a 2-3 week washout period where no supplements were ingested. After washout, the participants began the supplementation protocol with the opposing supplement and repeated the same experimental protocol as described above. KS plus MCT supplementation increased whole blood D-BHB concentrations above baseline measures 30 mins following supplement ingestion, with concentrations remaining elevated for a minimum of 120 minutes. At rest, whole blood D-BHB concentrations peaked at 0.79 + 0.9 mM 90 minutes following supplement ingestion. As hypothesized, twice-daily supplementation of KS plus MCT increased mechanical efficiency during constant load cycling at 70% VO_2max (2.82 ± 0.06 L/min vs. 3.14 ± 0.17 L/min, $p<0.05$)) and increased time to fatigue during the sub-maximal TTE protocol compared to placebo (33.42 ± 4.6 mins vs. 26.51 ± 4.3 mins, $p<0.05$). Resting (30 min following supplementation) and exercise measurements of whole blood BHB were significantly elevated in the KS condition, while serum glucose was not different between KS and placebo. Compared with placebo at rest and during constant load cycling at 50% VO_2max, serum insulin was significantly elevated (9.58 ± 2.5 mIU/L and 3.21 ± 1.8 mIU/L vs. 6.61 ± 1.7 mIU/L and 1.26 ± 0.4 mIU/L, $p<0.05$) and plasma glucagon was significantly reduced (33.76 ± 1.69 pg/mL and 124.22 ± 4.93 pg/mL vs 29.27 ± 2.52 pg/mL and 111.94 ± 8.29 pg/mL, $p<0.05$) in the KS plus MCT condition, suggesting that KS plus MCTs blunted the counter-regulatory response to

exercise. However, blood concentrations of cortisol, GH and lactate were not statistically different between conditions at all measurement time points of the TTE. Similar results were found with serum NEFA, although concentrations were significantly higher in the KS plus MCT condition at fatigue (1.87 ± 0.53 mMol/L vs. 1.22 ± 0.16 mMol/L, $p<0.05$). In contrast to the hypothesis, no differences in performance or mechanical efficiency were found between KS plus MCTs and placebo during the TT cycling protocol. Overall, these results indicate that 6 days of twice-daily supplementation of KS plus MCT may enhance prolonged, sub-maximal exercise performance but not moderate-to-high intensity exercise performance.

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Ac-CoA – Acetyl coenzyme A
AcAc – Acetoacetate
AcAc-CoA – Acetoacetyl-coenzyme A
ACAT – Acetyl-coenzyme-A-acetlytransferase
ATP – Adenosine-triphosphate
BDH - D-beta-hydroxybutyrate dehydrogenase
BDH1 - D-beta-hydroxybutyrate dehydrogenase 1
BHB – Beta-hydroxybutyrate
C8 – Caprylic acid
C10 – Capric acid
CHO - Carbohydrate
CIS – Canadian Interuniversity Sport
D-BHB – Beta-hydroxybutyrate (D Isoform)
L-BHB - Beta-hydroxybutyrate (L Isoform)
EDTA – Ethylenediaminetetraacetic acid vacutainer
EPI - Epinephrine
ETC – Electron transport chain
FFA – Free fatty acid
HCD – High-carbohydrate diet
HMGCL - 3-hydroxy-3-methylglutaryl-CoA lyase
HMG-CoA - Beta-hydroxy b-methylglutaryl-coenzyme A
HR – Heart rate
IMTAG – Intramuscular triacylglycerol
KB – Ketone body
KD – Ketogenic diet
KE – Ketone ester
KS – Ketone salt
MCFA – Medium-chain fatty acids
MCR – Metabolic clearance rate
MCT – Medium-chain triglyceride
MCT1 – Monocarboxylic acid transporter 1
mM - Millimole
NAD - Nicotinamide adenine dinucleotide (oxidized)
NADH - Nicotinamide adenine dinucleotide (reduced)
NE – Norepinephrine
NEFA – Non-esterified fatty acid
PDH – Pyruvate dehydrogenase
RER– Respiratory exchange ratio

SCOT - Succinyl-CoA:3-oxoacid-CoA transferase
SST – Serum separator tube
TAG - Triacylglycerol
TCA – Tricarboxylic acid
TTE – Time to exhaustion
TT – Time trial
VO_2 – Amount of oxygen inhaled
VO_{2max} – Maximal oxygen consumption

Chapter 1: Introduction

Since the beginning of competition, athletes have always looked to gain an edge over their competitors by sampling and implementing various physical, mental and spiritual interventions to maximize performance. With respect to physical intervention strategies, athletes have often paired various training practices with specific nutritional strategies to enhance their ability to perform and recover during periods of high volume training (Gould et al., 1999; Durand-Bush et al., 2002). Although much focus in the past was on maximizing an athlete's capacity to utilize carbohydrates and fat to fuel mechanical work, the recent commercial availability of exogenous ketone bodies has altered nutritional strategies within some elite athletes, placing a heavier focus on utilizing ketone bodies to fuel skeletal and cardiac cells (Evans et al., 2017). Likely, the shift to utilizing ketone bodies as a fuel source started as a result of the work of Veech et al., who demonstrated that the administration of exogenous glucose and ketone bodies in rodent hearts increased total cardiac output and reduced total oxygen consumption compared to glucose administration alone (Sato et al., 1995; Veech et al., 2001). This increased gross efficiency seen within perfused rat hearts prompted the use of a diet intervention that stimulated endogenous production of ketone bodies in hopes that athletes adhering to this diet, termed the ketogenic diet (KD) would increase gross efficiency during exercise performance. To date, it is still not fully understood as to whether the KD can improve exercise performance, as the benefit of ketone bodies may be limited to submaximal exercise intensities (Evans et al., 2017).

Despite this controversy, it is well established that long-term adherence to the KD is very difficult for athletes, particularly stemming from the severe restrictions placed on daily carbohydrate intake (Volek and Phinney, 2015). For this reason, the supplementation of

exogenous ketone bodies is an attractive nutritional strategy that may allow athletes to increase gross efficiency without drastically altering their current dietary habits (Evans et al., 2017). Specifically, a recent patent filed by D'Agostino et al., states that the co-ingestion of the ketone body beta-hydroxybutyrate (BHB) and medium-chain triglycerides (MCTs) caprylic (C8) and capric acid (C10) may be more beneficial than ingestion of ketone bodies alone, as a BHB and MCT supplement further increased blood BHB concentrations compared to BHB ingestion alone (D'agostino et al., 2017). Presently, there are no known studies reporting the effects of BHB and MCT during rest and exercise conditions within human subjects. Therefore, this thesis study aims to investigate the effects of BHB and MCT supplementation on whole body metabolism under resting and exercise conditions, and on exercise performance (Evans et al., 2017).

In Chapter 2, both endogenous and exogenous ketone body production and utilization will be reviewed comprehensively. This review will begin with the biochemical foundations of ketone body synthesis and the subsequent utilization by cardiac, hepatic, brain, skeletal and renal cells during resting conditions. The potential therapeutic role of ketone bodies during metabolic conditions such as fasting, carbohydrate restriction, disease and exercise will also be reviewed. Finally, the potential benefits of ketone bodies as an endogenous and/or exogenous fuel during exercise performance and competition will be examined.

Chapter 2: Biochemical Foundations and Review of Literature

Synthesis and Utilization of Ketone Bodies

When stressed by nutritional manipulations or physiological states that dramatically decrease CHO availability, mammalian species respond by stimulating an evolutionarily conserved adaptive response known as hepatic ketogenesis. Ketogenesis refers to the synthesis of four carbon, lipid-derived organ acids called ketone bodies (KB). Within mammalian species, three different forms of KB can be synthesized; namely acetoacetate (AcAc), D-beta-hydroxybutyrate (D-BHB) and acetone (Bergman, 1971; Veech et al., 2001; Evans et al., 2017).

Two key factors that stimulate the downstream production of ketone bodies, are: 1) elevations in the circulating glucagon-to-insulin ratio; and 2) reductions in hepatic glycogen. When either of these physiological conditions persist, lipolysis is accelerated, increasing the hepatic uptake and flux of fatty acids through B-oxidation. As the production of beta-oxidation-derived acetyl-CoA (Ac-CoA) exceeds the activity of citrate synthase and availability of oxaloacetate within the tricarboxylic acid (TCA) cycle, ketogenesis is stimulated. This is often viewed as a spillover pathway where excess Ac-CoA molecules not used within the TCA cycle are instead directed towards a fate-committing series of sequential reactions to produce ketone bodies (Evans et al., 2017; Puchalska and Crawford, 2017). The fractional conversion of FFA to ketone bodies is ~ 30% when blood [KB] is low, while conversion is ~85% when [KB] are high (Grey et al., 1975; Fery and Balasse, 1983, 1985, 1988).

The fate-committing reaction of ketogenesis begins with the condensation of Ac-CoA and acetoacetyl-CoA (AcAc-CoA) to produce beta-hydroxy b-methylglutaryl-CoA (HMG-CoA), which is then cleaved via 3-hydroxy-3-methylglutaryl-CoA lyase (HMGCL) to liberate Ac-CoA and AcAc (McGarry and Foster, 1977, 1980; Evans et al., 2017). From here, AcAc has 3 major

fates: 1) reduction via D-beta-hydroxybutyrate dehydrogenase (BDH) to form D-BHB; 2) spontaneous decarboxylation to form acetone; or 3) transport into circulation. The exact mechanism by which AcAc and D-BHB are transported across the inner membrane of mitochondria is not known, but it is widely accepted that AcAc and D-BHB are transported out of the hepatocytes into the circulatory system through the solute ligand carrier (SLC) protein 16A (SLC16A) family of monocarboxylate transporters (MCTs) (Fery and Balasse, 1989; Owen et al., 2002; Kahn et al., 2005).

Blood KB concentrations are a direct reflection of the balance between hepatic production of KBs (ketogenesis) and extra-hepatic terminal oxidation of KBs (ketolysis). Since AcAc is reduced to BDH in a NAD/NADH-coupled near-equilibrium reaction, the direct proportion of AcAc to D-BHB is reliant on the mitochondrial ratio of oxidized to reduced nicotinamide adenine dinucleotide (NAD/NADH). Metabolic stresses such as caloric restriction, prolonged exercise, alcohol consumption and/or low CHO availability increase the [NADH] within liver mitochondria, thus hepatic reduction of AcAc to D-BHB is favoured during these conditions. This reduction is reflected in KB appearance in circulation, as D-BHB:AcAc ratios can range from 1:1 up to 10:1, whereas acetone is generally in a 1:1 ratio with AcAc during times of caloric-restriction (Sokoloff, 1973; Siegel and McDonald, 1977; Robinson and Williamson, 1980; Cobelli et al., 1982; Veech, 2004; Cahill, 2006).

Following transport into the circulatory system, KBs have three primary fates: 1) terminal oxidation within extra-hepatic tissues; 2) integration into anabolic pathways to produce lipid and cholesterol molecules; or 3) excretion into the urine or lungs primarily in the KB form of acetone. Of these three fates, KBs are predominantly used as an energy substrate within the brain and skeletal muscle; however, KBs may also be utilized within liver, heart, kidneys, lungs and

the adrenal glands. Utilization by these peripheral tissues is facilitated by the transport of KBs from circulation into extra-hepatic tissues via MCT1/2 (Hagenfeldt, 1979; Beylot et al., 1986; Hasselbalch, et al., 1995; Veech, 2004; Cox and Clarke, 2014; Egan and D'Agostino, 2016; Puchalska and Crawford, 2017).

It is not fully understood how acetoacetate and D-BHB enter the matrix of extra-hepatic mitochondria, though it is believed that acetoacetate and D-BHB may use a combination of the mitochondrial pyruvate carrier (MPC) as well as diffusion to cross the inner mitochondrial membrane. Further research using UK 5099, a MPC inhibitor, has shown little effect on uptake of D-BHB into the mitochondria matrix, suggesting that D-BHB most likely diffuses across the inner membrane in the free acid form (Halestrap, 1975, 1978; Pande and Parvin, 1978; Puchalska and Crawford, 2017). Upon entry into mitochondria, molecules of D-BHB are re-oxidized to AcAc via 3-hydroxybutyrate dehydrogenase 1 (BDH1). Following oxidation, succinyl-CoA:3-oxoacid-CoA transferase (SCOT) catalyzes a covalent activation of AcAc from AcAc-CoA, while Ac-CoA acetyltransferase (ACAT) catalyzes a thiolase reaction of AcAc-CoA to produce two molecules of Ac-CoA to be used within the TCA cycle for terminal oxidation (Cohen and Stark, 1938; Saudubray et al., 1987; Fukao and Mitchell, 2004; Grabacka et al., 2016; Morris, 2016).

Stimulating Hepatic Ketogenesis Through Diet Manipulation

When the human body is challenged with physiological states such as starvation, fasting, adherence to a low-carbohydrate diet, prolonged exercise and/or pregnancy, KBs can play an integral role in providing the brain and other metabolically active tissues with an alternative fuel source to glucose. Unlike FFA, these lipid derived molecules have the ability to cross the blood brain barrier to provide an alternate energy substrate for the brain to oxidize during times of

extreme metabolic stress. Based on this unique capability, a predominant advantage of ketogenesis is that it provides a useable metabolic fuel from adipose tissue (as reviewed in Puchalska and Crawford, 2017).

To classify the degree of hyperketonemia (synonymous with the term ketosis), measurements of serum [D-BHB] serve as the primary quantitative marker. Based upon these concentrations, a non-diabetic individual can be classified into 3 various states of ketosis; intermittent or acute, nutritional, or therapeutic. A healthy human being consuming a standard western diet may achieve D-BHB blood concentrations of 0-0.5 mM if they supplement with ketogenic-promoting fats (such as short-chain MCTs) or engage in high intensity endurance exercise where ketosis is needed to supply skeletal muscle with sufficient energy to continue exercise (Veech, 2004; D'Agostino et al., 2013; Volek and Phinney, 2015). Since KBs are only temporarily elevated within these physiological states, this is metabolically defined as achieving a state of "intermittent or acute ketosis" (D'Agostino et al., 2013).

With more severe nutritional restrictions, blood KB concentrations may increase to 0.5-3.0 mM, where a dieter is defined as achieving a state of "nutritional ketosis". Evidence suggests that achieving nutritional ketosis is a very rigorous and restrictive process that can require a participant to maintain their macronutrient compositions for a minimum of 7-10 days just to initiate endogenous production of ketones (Wheless, 2004; Keene, 2006; Rho, 2008). Unfortunately, there is much controversy surrounding the literature of KDs due to the failure to accurately assign ketogenic macronutrient compositions, measure blood ketone concentrations and allow time for the participants to optimally produce, transport and oxidize KBs (Phinney, 2004; Kesl et al., 2016). Many studies reporting the use of KDs have failed to measure blood concentrations of D-BHB to assess whether participants achieved nutritional

ketosis through sustained blood D-BHB concentrations of 0.5-3.0 mM (Phinney, 2004; Kesl et al., 2016; Puchalska and Crawford, 2017). These blood concentration measurements are important in order to assess a KD intervention, as one insulin spike following a meal could instantly shift a participant out of nutritional ketosis (Phinney, 2004; Keene, 2006). The absence of detailed literature within this area may be the reason many studies have labelled their participants as being in nutritional ketosis without fully understanding the time course for achieving nutritional ketosis. Due to the inability to assess whether participants within a KD group actually achieved ketosis, many of the findings within short-term KD studies must be viewed with caution (Phinney, 2004; Keene, 2006; Kesl et al., 2016).

Nonetheless, when a strict KD is sustained for 7-10 days, endogenous ketogenesis is initiated, increasing the appearance of KB in circulation. Despite producing a state of hyperketonemia with this nutritional manipulation, a dieter must maintain a strict KD for another 7-14 days in order to achieve "keto-adaptation". Keto-adaptation refers to the metabolic energy shift where the favoured fuel source in brain and skeletal muscle cells shifts from glucose to KBs. The rate of this adaptation period is increased when carbohydrates are severely restricted. Though the optimum length of keto-adaptation is unknown, it is estimated to take a minimum of 2-3 weeks, following a strict KD (Phinney, 2004, Volek and Phinney, 2015).

During the keto-adaptation process, a higher reliance on KB for energy provision is proportionate to the presence and activity of ketogenic and ketolytic enzymes within metabolically active tissues. Specifically, this includes the increased presence and activity of ketogenic enzymes, BDH, ACAT and HMGCL in the liver, as well as the presence and activity of ketolytic enzymes, BDH1, SCOT and ACAT in extra-hepatic tissues (Veech et al., 2001; Puchalska and Crawford, 2017). The highest abundance of these ketolytic enzymes are within

brain, heart, kidney and skeletal muscle cells, while liver cells contain the lowest abundance, reflecting their priority to dispose KBs into circulation rather than utilize them for energy (McQuarrie and Keith, 1927; Fukao, et al., 1997, 2014). Of importance to this thesis, is the uptake of KBs by skeletal muscle during periods of rest and exercise. The uptake and utilization of KBs by skeletal muscle is primarily dependent on the appearance of KBs within circulation. When endogenously produced, circulating [KB] is determined by the rate of hepatic production of KBs, the rate of disposal into circulation and the extraction of KBs from circulation by active tissues (synonymous with the metabolic clearance rate (MCR) of active tissues) (Fery and Balasse, 1983; Balasse and Fery, 1989). During resting conditions, skeletal muscle MCR demonstrates curvilinear kinetics with dramatically increased extraction rates occurring at [KB] between 0 to 1 mM, while extractions rates between [KB] of 1 to 7 mM begin to steadily decrease. With these extraction kinetics, muscle accounts for ~50% of total KB extraction from circulation when blood [KB] are < 1mM, whereas muscle accounts for < 5% of total extraction when blood [KB] reach ~5 mM. The dramatic reduction in MCR indicates that KB extraction is a saturable process in skeletal muscle, with saturation being estimated between 1-2 mM (Fery and Balasse, 1983, 1985).

When carbohydrate and protein intake is severely restricted, as in the case with extended fasting; a dieter may achieve blood KB concentrations of 3.0-7.0 mM. When this metabolic state is stimulated, a dieter would be defined as achieving a metabolic state of “therapeutic ketosis” (Fery and Balasse, 1983; Cahill and Veech, 2003; Veech, 2004; Puchalska and Crawford, 2017). Within healthy, non-diabetic individuals, achieving [KB] above therapeutic levels is limited by the anti-lipolytic and insulinotropic effects of KBs themselves. This negative feedback loop is instrumental in regulating the rate of lipolysis and ketogenesis to preserve potential energy stores

needed during times of full starvation. As hepatic ketogenesis increases blood [KB] > 2mM, the secretion of insulin is stimulated, increasing hepatic uptake of KBs while proportionally inhibiting lipolysis (Fery and Balasse, 1983, 1985). This in turn reduces flux through B-oxidation, decreasing the hepatic mitochondrial Ac-CoA pool. Three-carbon TCA cycle intermediates and KBs themselves are both anti-ketogenic; therefore, reducing TCA cycle activity and increasing hepatic [KB] will synergistically attenuate the rate of ketogenesis (Hegardt, 1999; Fernandez-Figares, et al., 2004). Conversely, when individuals are unable to secrete insulin endogenously, blood KB concentrations can rise to ~20 mM, achieving a critical metabolic state defined as "diabetic ketoacidosis". In extreme cases, if an individual is not treated within several hours, diabetic ketoacidosis can become fatal (Cahill and Veech, 2003; Veech, 2004).

Therapeutic Benefits of Stimulating Hepatic Ketogenesis Through Diet Manipulation

Prior to clinical use, ketogenesis was dominantly viewed only to be advantageous during periods of calorie restriction and starvation where the human species required an alternate energy substrate to glucose to maintain survival (Grey et al., 1975; Keene, 2006]. Not until therapeutic ketosis ([KB] between 3-7mM) was established within epileptic patients did this classical view change. The first cause of epilepsy was believed to stem from intestinal intoxication where the body would absorb harmful toxins in the intestines during the digestion of a meal (Freeman and Vining, 1992; Freeman et al., 1998; Schwartzkroin, 1999). To mitigate this response, health practitioner Hugh Conklin prescribed a diet of only water for 30 days to patients with severe seizure occurrence. This fasting diet was not easy for patients to adhere to, though patients who completed the 30 days showed significant signs of improvement and decreased seizure occurrence (McQuarrie and Keith, 1927; Schwartzkroin, 1999; Keene, 2006). The success of this

diet was believed to be a direct effect of the high concentration of ketones that were produced during the water fast. Therefore, to produce similar effects and create a sustainable diet for epileptic patients, an alternative diet was created to promote hepatic ketogenesis. Initially, this diet, termed the ketogenic diet (KD), prescribed that ~80% of total calorie intake should come from fat, with the remaining 15% and 5% of total calories coming from protein and CHO, respectively (Veech, 2004; Keene, 2006).

To study the effectiveness of this KD, 150 epileptic children, with an average of 400 seizure episodes per month, were recruited. Of the total group, 30% had decreased seizure occurrence by 90%, while 3 subjects were completely seizure free while adhering to the diet (Freeman et al., 1998). It should be acknowledged that sufficient benefits of the KD were only seen when patients sustained blood D-BHB levels of ~4mM and restricted carbohydrate intake to <20 g/day (Freeman et al., 1998; Keene, 2006). When patients managed to adhere long-term they were also able to benefit for life, as Freeman reported that after 2 years on the KD, patients could resume eating a normal diet without seizure recurrence (Freeman et al., 1998). The exact mechanism by which the KD tempered the occurrence of seizures is still unclear. However, this research on the therapeutic effects of KD in epileptic patients opened up many lines of investigation on the potential beneficial effects of KDs for human health and performance.

Hepatic Ketogenesis and Skeletal Muscle KB Utilization During Exercise

Researchers speculated that KBs may have a metabolic and performance advantage over other skeletal muscle substrates such as glucose or FFA (Lardy, 1945; Lardy and Phillips, 1945; Kashiwaya et al., 1994; Veech, 2004). In support of this view, Veech et al. showed that perfused, isolated rat hearts that were administered D-BHB, AcAc and glucose had an increased cardiac output and decreased oxygen consumption when compared to rat hearts administered only

glucose (Sato et al., 1995; Veech et al., 2001). This suggests that KBs plus glucose are more metabolically efficient as a fuel source when compared to glucose alone. The increased efficiency is due to a larger Gibbs free energy (ΔG) or potential energy created within the electron transport chain (ETC) when KBs are oxidized. The larger ΔG was found to be a consequence of a more reduced NAD/NADH and more oxidized coenzyme-Q (CoQ/CoQH2) redox couple within the ETC. The change in potential energy at each of these sites increased the redox span between complex I and complex III which in turn increased the potential energy within the proton gradient (Masuda et al., 1990; Kim et al., 1991; Sato et al., 1995; Veech et al., 2000, 2001; Veech, 2006). The energy stored within the third phosphate bond during ATP synthesis is reflective of the potential energy within the proton gradient and as a result D-BHB, AcAc and glucose produced a ΔG of ATP hydrolysis of -57.6 kJ/mol compared to -56.2 kJ/mol with glucose alone. This increase in ΔG can be explained by the higher inherent energy within D-BHB compared to glucose, as D-BHB would liberate 243.6 kcal/mol of C_2 units compared to 223.6 kcal/mol of C_2 units if burned in a bomb calorimeter (Kashiwaya et al., 1994; Sato et al., 1995; Veech et al., 2001).

In addition to the changes measured within the mitochondrial matrix, rat hearts perfused with D-BHB, AcAc and glucose also increased cytosolic ATP concentration by 157% compared to rat hearts perfused with only glucose (Kashiwaya et al., 1994; Sato et al., 1995). The combination of increased potential energy within the proton gradient, higher inherent energy within D-BHB and a higher concentration of cytosolic ATP suggests that D-BHB and AcAc may also be two favourable energy sources for skeletal muscle during rest and exercise (Kashiwaya et al., 1994; Sato et al., 1995, Veech, 2004, 2006). As alluded to earlier, many studies using short-term (< 7-14 days) KD interventions have failed to produce adequate levels of hyperketonemia

(>0.5 mM) in participants and thus, should be viewed with caution (Phinney, 2004; Wheless, 2004). One exception to this is the short-term use of fasting. Severe caloric restriction can successfully produce [KB] > 0.5 mM within 24 hours, while prolonged fasts lasting 3-5 days have been shown to produce [KB] between 5-7 mM. Although this diet may not be ideal for exercise performance, the high concentration of circulating KBs make this nutritional manipulation a valuable tool in providing insight on how skeletal muscle utilizes KBs during periods of exercise (Fery and Balasse, 1983, 1985; Cox and Clarke, 2014).

After subjecting individuals to an overnight fast producing [KB] of ~ 0.2 mM, Balasse & Fery have shown that skeletal muscle accounted for ~50% of ketone extraction from circulation (Fery and Balasse, 1983, 1985). With the onset of a 50% VO_2max walking workload achieved on a treadmill, contracting muscle increased total KB extraction, accelerating MCR by ~40%. Though MCR decreased mildly throughout the duration of exercise, this intervention highlighted the ability for exercise to stimulate extraction of KBs by active skeletal muscle (Balasse and Fery, 1989). Conversely, when subjects underwent 5 days of fasting, producing an average [KB] of 5.5 mM, low intensity exercise did not significantly change MCR from rest; during the entire 2 hour exercise protocol, MCR remained steady indicating that skeletal muscle uptake was most likely saturated and could not be further stimulated by an increase in energy expenditure (Fery and Balasse, 1983, 1985).

Independent of nutritional status, exercise itself may stimulate hepatic ketogenesis in order to efficiently supply skeletal muscle with energy during exercise and recovery. When exercise is sustained for periods of time > 60 min, [KB] may increase up to ~0.3-2.0 mM for several hours during and following the termination of exercise (D'Agostino et al., 2013; Volek and Phinney, 2015). To increase KB utilization during these temporary metabolic states, the

activity of ketolytic and ketogenic enzymes within extra-hepatic tissues may be increased. Exercise-induced changes in ketolytic and ketogenic enzyme activity have yet to be fully determined within human subjects, though the effect of exercise on these enzymes within rodent models has been reported (Winder et al., 1974; Winder et al., 1975; Askew et al., 1975; Beattie and Winder, 1984; El Midaoui et al., 2006). As a whole, it has been established that trained rodents utilize KBs to a greater capacity than their sedentary counterparts, as noted by increased oxidation rates of D-BHB and AcAc within homogenates of trained muscle versus untrained muscle (Winder et al., 1975; El Midaoui et al., 2006). Increased KB oxidation has also been accompanied by higher enzyme levels of SCOT, ACAT and BDH1 following various running programs, with ketolytic enzymes showing the greatest increases within oxidative muscle, both slow and fast (Winder et al., 1974; Winder et al., 1975; Askew et al., 1975; Beattie and Winder, 1984). Altogether, the activity of ketolytic enzymes within rodent models suggests that KB oxidation may be enhanced within human subjects that are aerobically trained and genetically pre-disposed to a higher percentage of type I and IIA fibres (Evans et al., 2017).

Keto-adaptation and the Potential Performance Benefits of Hyperketonemia

As discussed previously, reports of the effectiveness of the KD on exercise performance must be viewed with caution, as many previous studies have failed to assess blood [KB] and did not provide sufficient time for keto-adaptation to occur. This is problematic because if participants have not completely shifted over to using KBs as a primary fuel source for the brain and skeletal muscle, exercise performance may actually be hindered. This principle was demonstrated in a study by Phinney et al., where subjects consuming a standard western diet were asked to sustain a KD for 6 weeks to determine if elevated circulating ketones could alter metabolic efficiency during a constant load, submaximal walking test completed on a treadmill

(Phinney et al., 1980). After the first week of the KD, at 65% of a participant's VO_{2max}, oxygen uptake was decreased by ~0.161 L/min, average heart rate decreased by ~5 bpm and duration to exhaustion decreased by ~ 38 min compared to the baseline diet. Following the full 6-weeks, oxygen uptake was decreased by ~0.378 L/min, average heart rate decreased by ~29 bpm and duration to exhaustion increased by ~ 81 min compared to the baseline diet. These findings suggest that mechanical efficiency can be increased when strict adherence to a KD is maintained for a minimum of 6-weeks.

In line with this principle, McSwiney et al. (2018), reported that performance advantages induced by the KD may only be achieved within elite competition after a competitor has adhered to a strict KD for a minimum period of 4 weeks. They showed that when elite cycling athletes switched from a high CHO diet (HCD) to a 4-week KD diet, power output during a time trial cycling protocol improved compared to the control group of elite cyclists sustaining a HCD (McSwiney et al., 2018). Though limited studies exist with respect to the effects of long-term adherence to a KD on exercise performance, these two studies indicate that potential exercise performance advantages may only be produced with strict adherence to a KD for a period of 4-6 weeks (Evans et al., 2017; McSwiney et al., 2018). When a KD is sustained for less than 4-6 weeks, exercise performance may actually be hindered despite significantly elevating blood [D-BHB] to >1.0 mM. Using elite race walkers, Burke et al., (2016), reported that a low-carbohydrate, KD increased VO_2 during a 10 km race walk and attenuated improvements in race performance during a 3-week intensive training intervention. Therefore, maintenance of a strict KD for a minimum of 4 weeks may be needed in order to improve performance during submaximal and real-life race performance exercise tests.

Importantly, long-term adherence to a KD diet has only been shown to improve exercise performance during sub-maximal workloads when aerobic respiration is almost solely relied upon for ATP production (Phinney et al., 1980; Phinney, 2004; McSwiney et al., 2018). Previous research suggests that KD diets may not be beneficial when anaerobic glycolysis is almost solely relied upon to fuel exercise performance, therefore, long-term adherence to a KD diet is speculated to be most beneficial for athletes completing endurance and ultra-endurance performances where VO_2 is not a limiting factor to maintain and/or continue exercise (Phinney et al., 1980; McSwiney et al., 2018). Despite potential improving exercise performance, long-term adherence to a strict KD may be difficult for individuals based on the severe restrictions to daily CHO intake allowance (Phinney, 2004; Evans et al., 2017; McSwiney et al., 2018). To avoid severely restricting dietary macronutrient compositions, one potential way to accelerate the keto-adaptation phase would be through the ingestion or administration of exogenous D-BHB (Volek and Phinney, 2015; Evans et al., 2017, McSwiney et al., 2018). Currently, exogenous ketones supplemented as either D-BHB ketone salts (KS) or ketone esters (KE), have been shown to increase blood [D-BHB] to >1 mM for up to ~0.5-6 hours regardless of dietary intake (Clarke et al., 2012; Cox et al., 2016; Shivva et al., 2016; Pinckaers et al., 2017). Accordingly, daily consumption of exogenous ketones may reduce the time needed for keto-adaptation as subjects could rapidly mirror a metabolic state that would be equivalent to the endogenous ketone production that may be seen around day 7-10 of a KD intervention (Wheless, 2004). The amount of time needed to upregulate ketolytic enzymes for optimal use of exogenous ketones is unknown but evidence from fat adaptation studies suggests that 5 days may be sufficient (Goedecke et al., 1999; Burke et al., 2000; Carey et al., 2001; Burke and Hawley, 2002; Burke et al., 2002; Burke and Kiens, 2006). For example, Goedecke et al. (1999) reported that when

participants were exposed to a high fat diet for 15 days, fat oxidation significantly peaked at day 5 and remained consistent for the following 10 days during exercise. These observations are consistent with other exercise studies that showed 5 days of high fat feeding were sufficient to significantly increase fat oxidation and reduce CHO oxidation when compared to a high CHO feeding control (Langfort et al., 1996; Burke et al., 2002; Burke and Kiens, 2006). A shift in substrate utilization from CHO to fat can be favorable for athletes training at low-moderate intensities, but fat adapting an athlete does come with consequences as studies have reported decreased activity of key CHO oxidation enzymes after only five days of high fat feeding (Langfort et al., 1996; Burke et al., 2000; Carey et al., 2001).

Interestingly, the reduced activity of CHO oxidation enzymes may not be as detrimental to performance during moderate-high intensity exercise when blood [D-BHB] is significantly elevated (Cox et al., 2016, Evans et al., 2017). During exercise loads that pre-dominantly favour CHO oxidation via glycogen utilization, D-BHB salvaged from KE sources may hold hierarchical preference over FFA and CHO during states of nutritional ketosis. Cox et al. (2016) reported that following KE ingestion, intramuscular [D-BHB] and acetyl-carnitine concentration increased ~300% and ~700% during high-intensity exercise. Intriguingly, these intramuscular changes persisted even without significant alterations in TCA cycle intermediates. Furthermore, when exercising at 75% Wmax, co-ingestion of KE with CHO increased intramuscular oxidation of D-BHB and TAG compared to CHO ingestion alone. Despite a decreased reliance on glycolysis, reflected by significantly decreased concentrations of glycolytic intermediates as well as decreased utilization of intramuscular glycogen, participants improved performance by ~2% during the 30-min time trial. Throughout the exercise protocol, no differences were seen with

[cortisol] and [insulin], though [FFA] and [lactate] were significantly reduced in the KE + CHO drink compared to CHO alone (Cox et al., 2016).

Although a few studies have shown that exogenous KBs can improve exercise performance during highly glycolytic workloads, it is important to note that the majority of performance outcomes during intensities of 80% VO_2max or higher have been impeded following fat adaption or acute doses of KSs and KEs (Burke and Kiens, 2006; Cox et al., 2016; Short, 2017; Evans et al., 2018; Waldman et al., 2018). Performance impedance within these studies was speculated to be a direct consequence of inhibition of skeletal muscle pyruvate dehydrogenase (PDH), thus performance benefits following long-term supplementation of exogenous ketones may be limited to exercise work intensities that are < 80% of an athletes VO_2max, where PDH activity is not heavily relied upon (Burke and Kiens, 2006; Cox et al., 2016; Evans et al., 2018).

The Physiological Response to Exogenous Ketosis During Rest and Exercise

Of the two exogenous D-BHB sources that currently exist, KE may be a more favourable ergogenic aid for their inherent ability to produce and sustain high blood [D-BHB] (>2 mM) compared with KS supplements (Clarke et al., 2012; Cox et al., 2016; Shivva et al., 2016). Unfortunately, KE supplements are not presently commercially available; therefore, the exogenous ketone source used within this study is KetoOS, a KS supplement that buffers the free acid form of D-BHB with sodium, magnesium, potassium and calcium salts (KetoOs, 2018). Despite the recent popularity, research regarding the pharmacokinetics of KS ingestion during rest is relatively novel, with only one known study comparing the post-absorptive metabolic response of both KS and KE during resting conditions in human participants (Stubbs et al., 2017). To analyze the differences between these two exogenous sources, a randomized, cross-

over design was used, where participants consumed 3.2 mmol/kg of exogenous D-BHB in the form of either 1) a sodium/potassium BHB salt, or 2) a KE (R-3-hydroxybutyl-R-1,3-hydroxybuyrate). Following KS ingestion, blood [D-BHB] peaked at 1.0 $\pm$ 0.1 mM within 1.5 hrs while [D-BHB] peaked at 2.8 $\pm$ 0.2 mM within 1 hr of KE ingestion. D-BHB concentrations remained elevated for ~4 hours with both ketone drinks, following non-linear, first order elimination kinetics after peak concentrations were reached. Notably, D-BHB AUC was ~30% lower with KS drinks compared to KE drinks; however, Stubbs et al. (2017) suggested the reason for this difference was likely due to the breakdown of BHB isoforms within each supplement. Unexpectedly, the KS supplement contained ~50% of the L-isoform while the KE drink contained over 99% of the D-isoform of BHB. Based on these results, it is speculated that whole-blood concentrations of [D-BHB] and subsequent utilization of D-BHB by peripheral tissue may be elevated if KS were to contain 100% of the D-isoform of BHB as seen within a KE supplement. Consequently, no known commercially available KS supplements to date are comprised of 100% D-BHB; therefore, all KS supplementation observations must take into the lower total ingestion of D-BHB when comparing to KE supplementation.

Furthermore, when taking whole blood [D-BHB] measurements, many studies favour the use of handheld ketone meters for their ease of use. However, ketone meters use enzyme-based reagent strips that exclusively measure the D-isoform of BHB (Kesl et al., 2016; Evans et al., 2017; O'Malley et al., 2017). Therefore, to distinguish differences in BHB isoforms within whole blood, Stubbs et al. (2016) analyzed venous blood samples and time matched these with their ketone meter measurements. Remarkably, analysis revealed that total [BHB] peaked at 3.4 + 0.2 mM (compared to original reports of 1.0 + 0.1 mM) with the L-isoform producing significantly higher concentrations at 1-4 hrs following ketone ingestion than concentrations of

the D-isoform. Additionally, while blood [D-BHB] returned back to baseline levels 4 hours after ingestion, [L-BHB] remained significantly elevated 8 hours post-ingestion, with blood concentrations returning to baseline levels after 24 hrs.

Concerning changes in blood metabolites, KS drinks significantly decreased FFA, TG and glucose concentrations between 0.5-3 hours post-ingestion, with metabolite concentrations returning to baseline at 4 hrs. Plasma insulin was also significantly increased 30 min following KS consumption, but no statistical differences from baseline were found 60 min post-ingestion. Additionally, the amount of BHB excreted in the urine during KS ingestion was also negligible, accounting for <1.5% of total BHB ingested (Stubbs et al., 2016). Based on these observations, the loss of BHB to the external environment through the urine is not a major concern for participants consuming exogenous KS, with ingested BHB molecules remaining within circulation for a minimum of 4 hours.

With reference to the effects of exogenous ketosis during exercise performance, throughout the literature it has been cited that an improved energetic efficiency has been seen within rodent heart during acute exogenous ketosis (Sato et al., 1995; Veech, 2004, 2006). Despite the inherent energetic efficiency within molecules of D-BHB, establishing and measuring the improved mechanical efficiency following KS ingestion is one area of research that still needs more focus (as reviewed in Evans et al., 2017). In theory, if exogenous ketosis improved energetic efficiency, this would be measured by a higher power output at the same oxygen consumption rate as control or by a lower oxygen consumption rate at the same power output as control (Cox et al., 2016, Evans et al., 2017). To measure these two different performance scenarios, a constant, sub-maximal load protocol or time trial protocol could be

completed during exogenous ketosis to distinguish if an energetic efficiency advantage exists compared to control (Cox and Clarke, 2014, Cox et al., 2016).

Among the very limited known studies using KS ingestion prior to exercise, conflicting oxygen uptake results exist during constant load exercise, with studies reporting increased and negligible changes in VO_2 compared to control (Rodger et al., 2017; Evans et al., 2018). Moreover, stationary bike time trial protocols using highly glycolytic intensities (> 75% VO_2peak) have also shown conflicting results, with KS either increasing, decreasing or sustaining comparable performances to control (Rodger et al., 2017; Short, 2017; Evans et al., 2018; Waldman et al., 2018). Blood [lactate], [FFA] and [insulin] during exercise following exogenous ketosis were also conflicting among these studies, with the only similar metabolic response to KS ingestion being a decrease in [glucose] at pre and post-exercise time points (O'Malley et al., 2017; Rodger et al., 2017; Short, 2017; Evans et al., 2018; Waldman et al., 2018).

These conflicting results may be attributable to individuals being responders vs. non-responders to KB, though it is also important to take into account the current metabolic state of each individual (Sparks, 2017;Williamson et al., 2017). Depending on specific dietary, exercise and non-exercise habits, the activity of ketolytic enzymes BDH1, SCOT and ACAT within skeletal muscle cells could be significantly different between age-matched individuals (Phinney, 2004; McSwiney et al., 2018). Therefore, to improve KB utilization and 'level out the playing field', exposing participants to repeated supplementation of KS may increase muscle ketolytic enzyme activity to a higher degree than current exercise and diet habits could possibly stimulate (Veech, 2004; Evans et al., 2017; McSwiney et al., 2018). Importantly, this is contingent on using participants who are not currently consuming a KD or practicing intermittent fasting,

where a significant upregulation of ketolytic enzyme activity would already be stimulated (Volek and Phinney, 2015; McSwiney et al., 2018).

Additionally, it is important to highlight that the ketone supplement used within this study, KetoOS, is unique to other KS supplements in that medium-chain triglycerides (MCTs) have also been added to the supplement formula (D'Agostino et al., 2017)]. MCTs refer to triglycerides that contain a 6-12 carbon backbone. Short-chain MCTs with 8 and 10 carbon backbones are favoured supplements for exercise due to their inherent ability to stimulate endogenous ketogenesis. When consumed separate of exogenous ketones, large doses of caprylic acid (C8) and capric acid (C10) can induce a state of diet-induced hyperketonemia, significantly raising blood [D-BHB] and [AcAc] above baseline concentrations (Bach and Babayan, 1982; Decombaz et al., 1983; Wood and Kelly, 2017). The increase in KB appearance within circulation is a direct reflection of the metabolic advantage that C8 and C10 inherently contain. Compared to triglycerides of greater molecular weight, these two molecules bypass lymphatic transport and are instead transported via hepatic portal circulation upon absorption (Bach and Babayan, 1982). This alternative absorption pathway may increase overall efficiency, providing the liver with acute, supra-optimal concentrations of Ac-CoA (Veech, 2004; Evans et al., 2017). As noted earlier, when the presence of Ac-CoA molecules exceeds the activity of citrate synthase and the presence of oxaloacetate, KB formation is stimulated (Bach and Babayan, 1982; Evans et al., 2017).

The stimulation of hepatic ketogenesis following MCT consumption may be the reason that co-ingestion of MCTs with KS has produced significantly higher blood [D-BHB]s when compared to KS ingestion alone. Within Sprague-Dawley rats, a single dose of a MCT + KS supplement significantly increased [D-BHB] to ~2 mM at 4-hours post-administration, while the

KS supplement alone peaked at ~ 0.4 mM. Moreover, following 1-week of daily administration, [D-BHB] peak increased to ~2.2 mM in the MCT + KS supplement group, while administration of KS produced identical concentrations to baseline measures (Kesl et al., 2016).

MCT's may be a potential performance aid during exercise that relies heavily on aerobic metabolism (Van Zyl et al., 1996). When MCTs are ingested alone, exercise performance has been decreased or unaffected compared to CHO ingestion, but when MCTs are combined with CHO, exercise performance has been increased or remained consistent compared to CHO ingestion (Jeukendrup et al., 1995, 1998; Goedecke et al., 1999; Angus et al., 2000; Wang et al., 2018). Based on these results and the results of Kesl. et al. (2016), many supplement companies have now added significant amounts of MCTs within their exogenous ketone supplements in hopes of improving hyperketonemia following ingestion and subsequent exercise performance (D'Agostino et al., 2017; KetoOs, 2018). Presently, in healthy humans, the pharmacokinetics and pharmacodynamics of the synergistic ingestion of KS and MCTs at rest and during exercise have not been reported (Evans et al., 2017). Accordingly, one aim of the present study was to assess the changes in plasma [BHB], [glucose], [non-esterified fatty acids] ([NEFA]) and [lactate] during resting and exercise protocols.

The Endocrine Response to Exercise

At the onset of exercise, it is well established that the sympathoadrenal system responds to an increase in energy demand by releasing both fast-acting and slow-acting hormones to aid in maintaining adenosine triphosphate (ATP) homeostasis within skeletal muscle cells (as reviewed in Terjung, 1979). Depending on the duration and intensity of exercise, skeletal muscle cells may counteract the rate of ATP degradation through uptake and terminal oxidation of extracellular energy substrates such as CHO and NEFA (Terjung, 1979; Grey et al., 1993). In order to

maintain euglycemia, the sympathoadrenal system stimulates the release of fast-acting hormones norepinephrine (NE) and epinephrine (Epi) from the adrenal glands, subsequently stimulating increased hepatic output of glucose via glycogenolysis and gluconeogenesis. To increase the availability of NEFA for utilization by skeletal muscle, NE and Epi also stimulate lipolysis within adipose tissue, increasing the release of NEFAs into circulation. Additionally, NE and Epi act upon the pancreas to reduce the release of insulin and increase the secretion of glucagon. The reduction in insulin concentration reduces insulin-dependent glucose uptake in peripheral tissues and augments liver glucose production and the increase in plasma glucagon stimulates both adipose tissue lipolysis and hepatic glucose production (Terjung, 1979; Cryer, 1980).

As exercise progresses, plasma glucose and NEFA concentrations are also maintained by the slow-acting hormones growth hormone (GH) and cortisol. These two hormones play a synergistic role alongside NE and Epi, stimulating hepatic glucose synthesis and liberation of NEFAs from adipose tissue. When exercise duration is prolonged, intracellular energy substrates such as intramuscular triacylglycerol (IMTAG) and glycogen may become limited, placing a heavier reliance on the uptake of extracellular substrates (Terjung, 1979). In order to maintain energy supply for skeletal muscle cells to continue exercise, NE, Epi, GH and cortisol are further elevated to increase the release of glucose and NEFA into circulation while simultaneously increasing the secretion of glucagon and reducing the secretion of insulin from the pancreas (Cryer, 1980).

During low-high intensity exercise, it is well documented that glucose concentrations within healthy adult males usually remain constant throughout exercise, however, hyperglycemia and hypoglycemia may be experienced when nutritional intake and exercise duration is manipulated (Terjung, 1979). In the case of diet manipulation, when systemic CHO availability

is low, an individual may experience a state of hypoglycemia during exercise (Cox and Clarke, 2014; Volek and Phinney, 2015). Within this metabolic state, hypoglycemia further stimulates the release of NE and Epi, increasing the hepatic uptake of NEFA liberated from adipose tissue (Terjung, 1979). As discussed previously, increasing hepatic uptake of NEFA can stimulate the synthesis of KBs, increasing concentrations of AcAc and D-BHB within circulation (Puchalska and Crawford, 2017). Although KB production is stimulated under hypoglycemic conditions during exercise, when glucose concentrations remain relatively constant throughout exercise, KB production is negligible (Evans et al., 2017).

The development of new exogenous ketone sources has directed research towards characterizing the metabolic response to exogenous ketosis during exercise (Cox et al., 2016; Evans et al., 2017). Recent studies have reported shifts in substrate utilization and enzyme activity following exogenous ketone ingestion (Cox et al., 2016; Stubbs et al., 2017). Hyperketonemia achieved via exogenous ketone ingestion has been shown to increase the reliance on terminal oxidation of KB in place of glucose and NEFA (Cox et al., 2016; Evans et al., 2017). Specifically, when KE and CHO were ingested prior to exercise, plasma concentrations of D-BHB were elevated while plasma concentrations of glucose, lactate and NEFA were reduced compared to CHO ingestion alone. Furthermore, following 120 min of cycling at 70% VO_2max, IMTAG concentrations were significantly reduced when KE and CHO were ingested prior to exercise compared to ingestion of CHO alone (Cox et al., 2016). Based upon these responses, KS and MCT ingestion would be expected to reduce the reliance on extra-cellular energy substrates and, therefore, result in a blunted endocrine response including decreased glucagon, cortisol and GH and increase insulin concentrations compared to placebo

(Terjung, 1979). However, the endocrine response following KS and MCT ingestion still remains unexplored in human subjects at present.

Research Purpose

In summary, studies investigating the effects of a KD on exercise performance have shown equivocal results, with some studies reporting increased exercise performance during submaximal constant load protocols and other studies reporting impeded performance during highly glycolytic, sprint protocols. Despite the potential for the KD to improve performance, it is well documented that long-term adherence to the KD is very difficult for many individuals. To achieve a state of hyperketonemia and bypass adherence to the KD, the supplementation of exogenous KBs may be a more plausible nutritional manipulation to sustain long-term. Exogenous KBs in the form of KEs have also been shown to improve performance during submaximal exercise, however, the effects of KSs and MCTs on submaximal exercise have yet to be reported. Therefore, the objectives of this study were to characterize the effects of exogenous KSs and MCTs on whole body resting and exercise metabolism and exercise performance in male varsity athletes. The pharmacokinetic and pharmacodynamic whole blood D-BHB response following a 5-day KS and MCT ingestion loading phase was also determined.

Objectives

1. Assess the pharmacokinetic and pharmacodynamic whole blood D-BHB response at rest following a single, fixed-volume dose of KS and MCT compared to placebo.
2. Assess whether the pharmacokinetic and pharmacodynamic whole blood D-BHB responses at rest following a single, fixed-volume dose of KS and MCT are altered after 5 days of twice-daily consumption of a fixed-volume dose of KS and MCT or placebo.
3. Characterize the plasma BHB and serum glucose response at rest and during a submaximal time to exhaustion (TTE) protocol following 6 days of twice-daily

consumption of a fixed-volume dose of KS and MCT.

4. Characterize the endocrine response at rest and during exercise following 6 days of twice-daily consumption of a fixed-volume dose of KS and MCT compared to placebo. To characterize the response, measurements of blood insulin, lactate, NEFA, cortisol, glucagon, and GH were taken during a TTE cycling test.
5. Assess the potential for 6 days of twice-daily consumption of a fixed-volume dose of KS and MCT to alter mechanical efficiency and exercise performance during a TTE cycling test compared to placebo. To assess changes in mechanical efficiency and exercise performance, measurements of oxygen uptake (VO_2), heart rate, RER, RPE and time to exhaustion were taken.
6. Characterize the plasma BHB and serum glucose response at rest and during a 10-km cycling time trial (TT) protocol following 8 days of twice-daily consumption of a fixed-volume dose of KS and MCT.
7. Assess the potential for 8 days of twice-daily consumption of a fixed-volume dose of KS and MCT to alter mechanical efficiency and exercise performance during a TT cycling test compared to placebo. To assess changes in mechanical efficiency and exercise performance, measurements of oxygen uptake (VO_2), heart rate, RER and exercise duration were taken.

Hypotheses

i) Whole blood D-BHB concentrations will be increased above 0.1 mM for 120 min following KS and MCT consumption compared to placebo.

ii) The pharmacokinetic and pharmacodynamic whole blood D-BHB response to a single, fixed-volume dose of KS and MCT will be similar before and after 5-days of twice-daily consumption of a fixed-volume dose of KS and MCT.

iii) Whole blood D-BHB concentrations will be increased during the TTE and TT exercise protocols following KS and MCT consumption compared to placebo.

iv) Venous blood concentrations of glucose, lactate, NEFA, GH, cortisol and glucagon will be decreased during the TTE exercise protocol following KS and MCT consumption compared to placebo.

v) Venous blood concentrations of insulin will be increased during the TTE exercise protocol following KS and MCT consumption compared to placebo.

vi) Venous blood concentrations of glucose will be decreased during the TT exercise protocol following KS and MCT consumption compared to placebo.

vii) VO_2, HR and RER will be decreased during the TTE and TT exercise protocols following KS and MCT consumption compared to placebo.

viii) Time to fatigue during the TTE protocol will be increased following KS and MCT consumption compared to placebo.

ix) Time to complete the TT protocol will be decreased following KS and MCT consumption compared to placebo.

Methods

Participants and Guidelines for Dietary Intake

Eight male University of Waterloo athletes were recruited to participate in this study. Of the 8 participants, 7 athletes competed within varsity-level ice hockey and 1 athlete competed within varsity-level cross-country running. All athletes were within the off-season phase of their competitive sports, with the eight participants completing on average 3.8 $\pm$ 0.3 resistance and/or aerobic training sessions of at least 60 min per week. Based on this volume of training, participants were asked to refrain from exercise for 24 hours before the start of each study phase. From day 1 to day 8 of each study phase, participants were asked to refrain from engaging in any forms of resistance and/or aerobic training. Following the final session (day 8) of the first study phase, the athletes were asked to return to their regular exercise schedules while they completed a 2-3 week washout period.

Additionally, participants were asked to complete a dietary journal, recording all caloric intake from day 1 to day 8 of the first study phase. Participants completed their dietary journals using a customized 8-day journal that categorized calorie intake into type, amount or volume, and time of consumption. Meals were not standardized the night before and morning of laboratory test dates. Therefore, participants were asked to repeat their dietary habits from phase 1 of the study, with a highlighted focus on consuming the exact same meals and beverages for the 24 hours prior to the commencement of laboratory testing on day 1, 5, 6 and 8 of phase 2 of the study.

Experimental Design

A single-blinded placebo-controlled crossover design was employed where participants were randomized to either KetoOS (0.3 g βHB/kg, 0.18 g MCT/kg 0.02 g Calcium/kg, 0.02 g

Sodium/kg and 0.02 g Potassium/kg, average calories per participant serving: 183.6 ± 0.9 kcal) or placebo (0.02 g Calcium/kg, 0.02 g Sodium/kg and 0.02 g Potassium/kg, average calories per participant serving: 0 kcal) (see Figure 1). Prior to the supplementation period, all participants completed a VO_2max test and two exercise familiarization sessions to get accustomed to the exercise protocols and testing procedures used in this study. Following this, the study commenced with a supplementation protocol where participants consumed a KetoOS or control supplement twice daily for a total of 8 days. On days 1 and 5 of supplementation, participants reported to the laboratory for blood collection to assess plasma [BHB] time course responses. The next day (day 6), participants completed a sub-maximal cycling test to exhaustion to allow for the examination of exogenous ketone ingestion on the endocrine response to exercise, mechanical efficiency and exercise performance. Forty-eight hours later (day 8), participants performed a 10-km cycling time trial protocol to further explore the effects of exogenous ketone ingestion during a moderate-high intensity exercise performance. Participants then completed a 2-3 week washout period where no supplements were ingested and participants were able to return to their normal activities of daily living. After washout, the participants began the supplementation protocol with the opposing supplement and repeated the same experimental protocol as described above. The participants were asked to refrain from caffeine consumption during the morning of their exercise tests and encouraged to drink sufficient levels of water to ensure they were properly hydrated. All participants were fully informed of all procedures and risks before giving written consent. Written ethics approval has been given for this study by the Office of Research Ethics at the University of Waterloo.

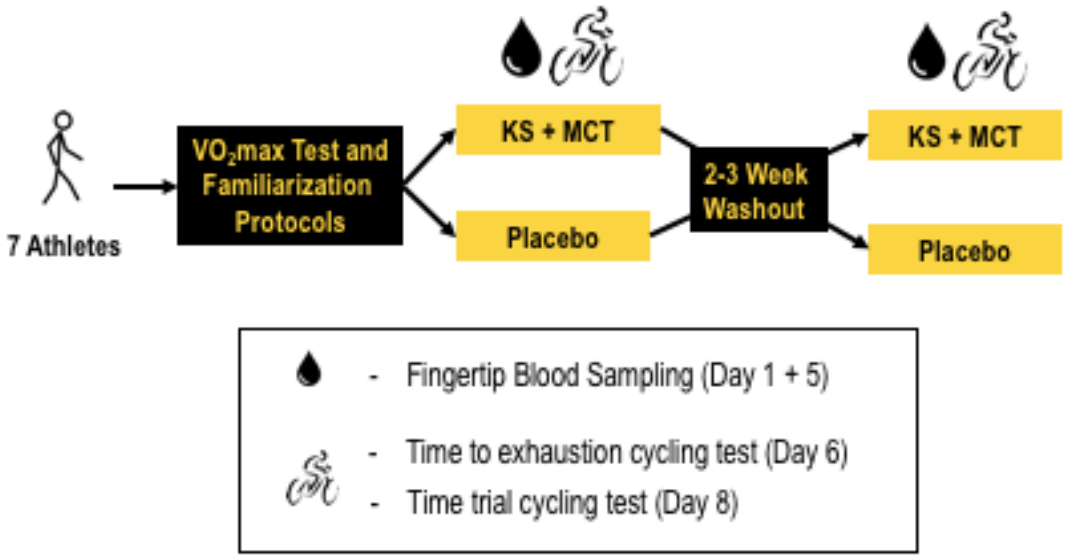

Figure 1. Experimental Design Time Course. A single-blinded placebo-controlled crossover design was employed where participants were randomized to either a ketone salt (KS) and medium-chain triglyceride (MCT) supplement or placebo. Seven male varsity athletes were recruited for this study, with participants completing two 8-day phases of either twice-daily supplementation of KS and MCT or placebo. Prior to initiating the first phase, participants completed a VO2max test and two familiarization sessions to get accustomed to the TTE and TT cycling tests. On day 1 and 5 of each study phase, participants reported to the laboratory for blood collection to assess whole blood [BHB] time course responses. On day 6, participants completed a sub-maximal test to exhaustion. Forty-eight hours later (day 8), participants performed a 10-km cycling TT protocol which was followed by a 2-3 week washout period where no supplements were ingested. After washout, the participants began the supplementation protocol with the opposing supplement and repeated the same experimental protocol as described above.

VO_{2max} Test

In order to calculate the work rates that were used for the time to exhaustion protocol, participants completed a stationary bike VO_{2max} test before the first phase of the study. The progressive exercise protocol included 2 minute stages at each work rate, with resistance increasing until the participant fatigued. The participant started with a work rate of 50 Watts and resistance was increased by 50 Watts for the next three stages. After the fourth stage was

completed, work rate only increased by 25 Watts for subsequent stages until fatigue was reached. Once the participant reached fatigue, the resistance on the bike was rapidly reduced and the participant was asked to cool down at a work rate of 50 Watts for several minutes. The VO_{2max} test was considered valid if the RER was $\geq$ 1.15 or the HR was within 10 beats/min of the age predicted HR_{max}. Once completed, the data were exported to Excel and used to create an equation for the line of best fit for VO_2/work rate. This equation was then used to calculate the 50 % and 70 % of VO_{2max} workloads for the time to exhaustion protocol for each participant.

Familiarization Trials

Participants were asked to complete two familiarization trials on two separate visits prior to the start of the supplementation trials. Familiarization trials included the time to exhaustion and time trial protocols (see below for details) that were used during the experimental trials. Participants did not consume any supplements prior to these trials and were not asked to arrive in a fasted state. No blood samples or Vmax system measurements were taken or recorded during the time to exhaustion or time trial familiarization protocols.

The TTE cycling protocol was completed on a ViaSprint 200P Ergoline stationary bike, which was uniquely designed to keep work rate constant despite the revolutions per minute performed by each participant during testing. Since this bike did not measure distance traveled, the TT cycling protocol was completed on a Keiser M3 Indoor Cycle. Visual displays on both stationary bikes were blinded from participants using black tape, and all visual cues for exercise duration (time) were removed from the laboratory.

KetoOS and Control Supplementation Protocols

As mentioned above, participants were randomized to either KetoOS or placebo In order to mask the taste of the drinks, 1 g of flavoured stevia (French Vanilla Zero Calorie Sweetener, NOW Nutrition) was added to each control supplement.

The supplementation protocol included the participants consuming KetoOS or control two times daily for 8 days. It was recommended to all participants to drink the first dose of control or KetoOS 30 minutes following their first meal and the second dose approximately 5 hours after the first dose was consumed. Due to sleeping schedule and meal time differences among participants, each participant was asked to record the times they consumed their own supplement drinks during phase one of the study so that they could consume the supplements during the same time in phase two. All dietary journals were handed in to the student investigator following phase one where they were copied and returned back to the participants following their 2-week washout period.

On days 1,5,6 and 8, participants consumed a standardized meal replacement bar (BioSteel Nutritional Bar, 170 calories, 5 g of fat, 22 g of CHO, 12 g of protein) 30 minutes prior to consuming their supplements. On these four days, participants arrived to the laboratory after fasting for a minimum of 3 hours and consumed the standardized meal replacement bar after preliminary blood samples were taken. Besides the fasting periods and controlled meals on days 1,5,6 and 8, participants maintained their current dietary patterns and ate *ad libitum* throughout the day.

Fingertip Blood Sampling and D-BHB and Glucose Measurements

On day 1 and 5, participants completed fingertip blood sampling in order to characterize the blood [D-BHB] response to ingestion of the control and ketone supplements (see Fig. 2).

Upon arrival, a fingertip blood glucose measurement was taken alongside a blood [D-BHB] measurement in order to determine if the participant was sufficiently fasted. After these preliminary measurements were taken, the participants consumed the standardized nutritional bars and a blood [D-BHB] measurement was taken 30 min later. Following measurement, the participants then consumed their supplement drinks and subsequent blood [D-BHB] measurements were taken 30, 60, 90 and 120 minutes following supplement ingestion. After participants completed the final fingertip blood sample, they were reminded of the time they ingested their first supplement dose and given the time that they should aim to consume their second supplement dose later that day.

Capillary blood [D-BHB] concentrations were measured using a ketone meter (Precision New, Abbot Laboratories, Witney, UK) and blood glucose concentrations were measured using a glucose meter (Truetrack Blood Glucose Monitoring, Midland City, US) . During assessment, the participants were asked which finger they would prefer to take a fingertip prick blood sample from and this finger was wiped clean with an alcohol swab by the student investigator. The finger was pricked using a sterile, single-use lancet device (Bayer Single-let Disposable Lancet, Whippany, US) that is commercially available. The prick was targeted approximately 1 cm from the tip of the finger, somewhere between the midpoint and side of the finger. The first drop of blood was wiped away using an alcohol swab and the subsequent blood sample was collected onto the blood ketone strip. If the quality of the sample was not sufficient enough to produce a measured value, then an opposing finger was pricked to achieve a value.

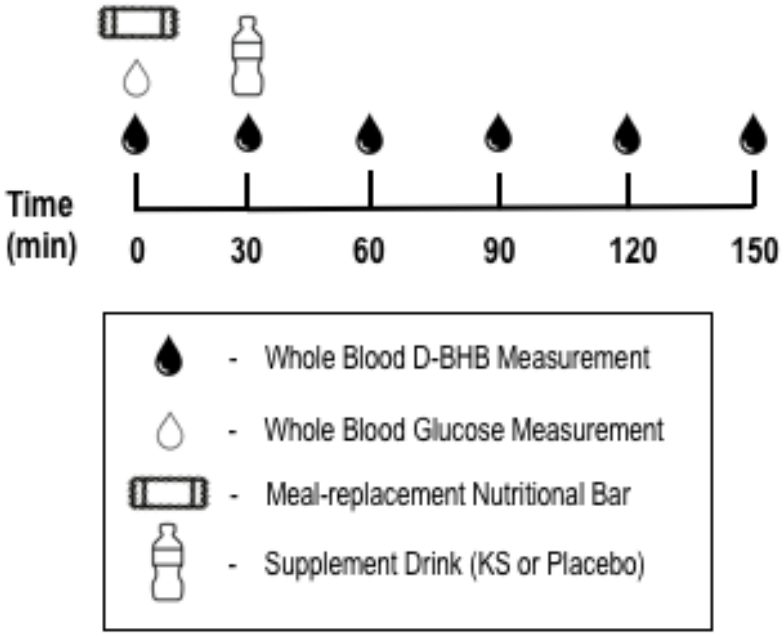

Figure 2. Fingertip blood sampling and D-BHB and glucose measurement time course. Participants arrived to the laboratory in a minimum 3-hour fasted state and whole blood glucose and D-BHB measurements were immediately taken. Participants consumed a meal-replacement nutritional bar following initial blood sampling. Whole blood D-BHB samples were then taken again every 30 mins for the following 2 hours (30-150 mins). Following the second blood sample (30 mins), a ketone salt (KS) or placebo supplement drink was consumed.

Time to Exhaustion (TTE) Protocol

Following 5 days of supplementation, participants reported to the laboratory on day 6 in a 3-hour fasted state (see Fig 3.). Preliminary venous and fingertip blood samples were taken upon arrival and participants consumed the standardized nutrition bar immediately after blood sampling. Thirty minutes later, the participants consumed the supplement drinks following another venous and fingertip blood sample. After the supplement was consumed, electrodes were then placed on the participants and the Vmax headgear was mounted. Exactly 15 minutes following supplement ingestion, the Vmax system began collecting resting data while the

participants were seated on a chair. Resting measurements were taken for a total of 15 minutes and venous and fingertip blood samples were taken with 5 minutes remaining in the measurement time period. Participants were asked to warm-up for a minimum of 5 minutes to a maximum of 15 minutes. The total warm-up time for phase one was recorded in order to repeat the same timing for phase two. The time to exhaustion bike protocol then commenced with the participants cycling at 50% of their VO_{2max} for 20 minutes. This workload was then followed by a work rate of 70% VO_{2max} that was maintained until exhaustion was reached. Venous and fingertip blood samples were taken 10 minutes into the 50% and 70% VO_{2max} workload and a final blood sample was taken immediately at fatigue. Participants were also asked to rate their perceived exertion levels 10 minutes into the 50% and 70% VO_{2max} workload using a Borg RPE Scale.

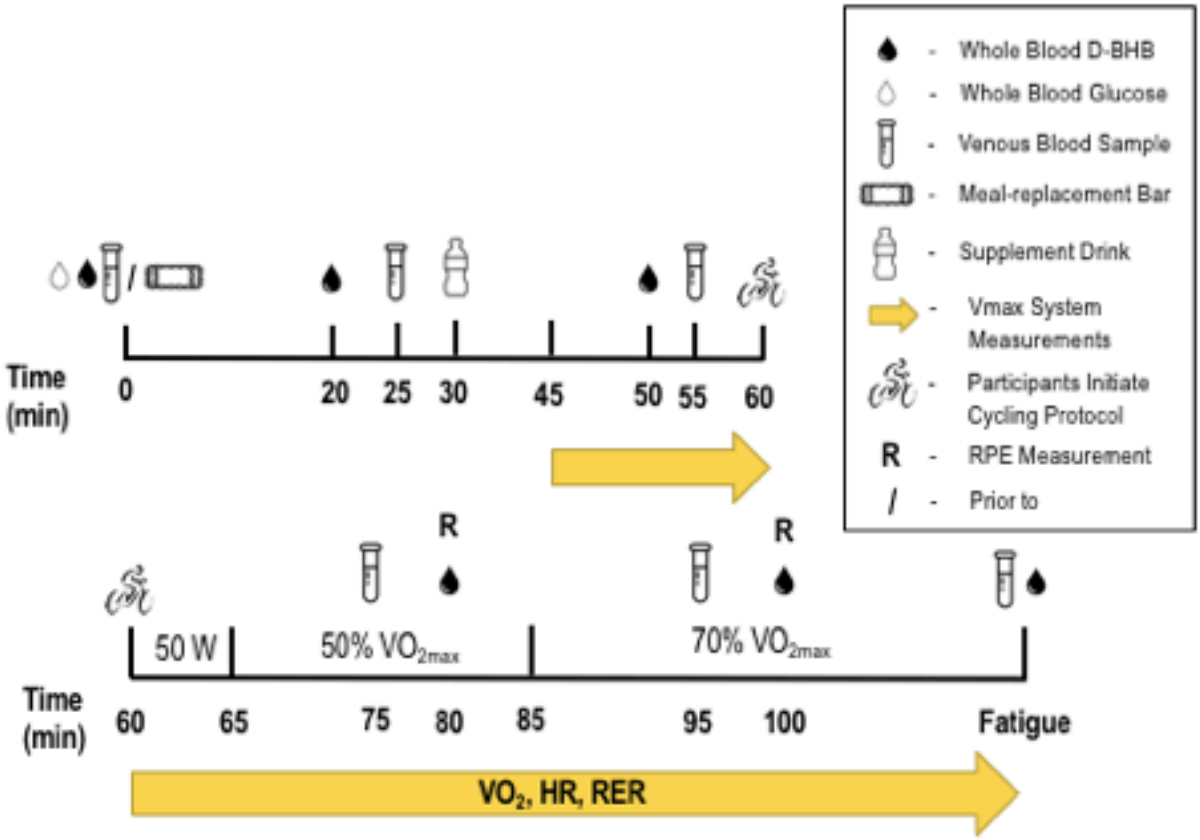

Figure 3. TTE Laboratory Test Protocol. Participants arrived to the laboratory in a minimum 3-hour fasted state. Upon arrival, fingertip and venous blood samples were taken. A meal-replacement bar was consumed immediately following blood sampling and the supplement drink (KS or placebo) was ingested 30 mins later. Venous and fingertip blood measurements were taken again prior to supplement drink ingestion, initiation of the time to exhaustion (TTE) cycling protocol, during the 50% VO2max workload, during the 70%VO2max workload and once a participant reached fatigue. To collect resting and exercise oxygen uptake (VO2), heart rate (HR) and respiratory exchange ratio (RER) data, Vmax system measurement collections began 15 mins prior to exercise initiation and were terminated at fatigue. Rate of perceived exhaustion (RPE) measurements were taken during the 50% VO2max and 70% VO2max workloads of the TTE test.

Time Trial (TT) Protocol

Forty-eight hours after completing the time to exhaustion protocol, participants arrived at the laboratory on day 8 to complete a time trial (see Fig. 4). Participants were asked to complete a 10-km distance in the fastest time possible using a self-selected stationary bike resistance. This

bike test mirrored the time to exhaustion protocol from participant arrival to stationary bike warm-up but replaced the two submaximal workloads with a single workload. After completing the bike warm-up, participants were asked to refrain from pedaling to allow the odometer to reset to 0 km. Once reset, the participants started from a complete stop and continued pedaling until the 10-km was reached. At 4.5 km and 9.5 km of the time trial, venous and fingertip blood samples were taken. During these distance points, participants were also asked to rate their perceived exertion levels using a Borg RPE Scale.

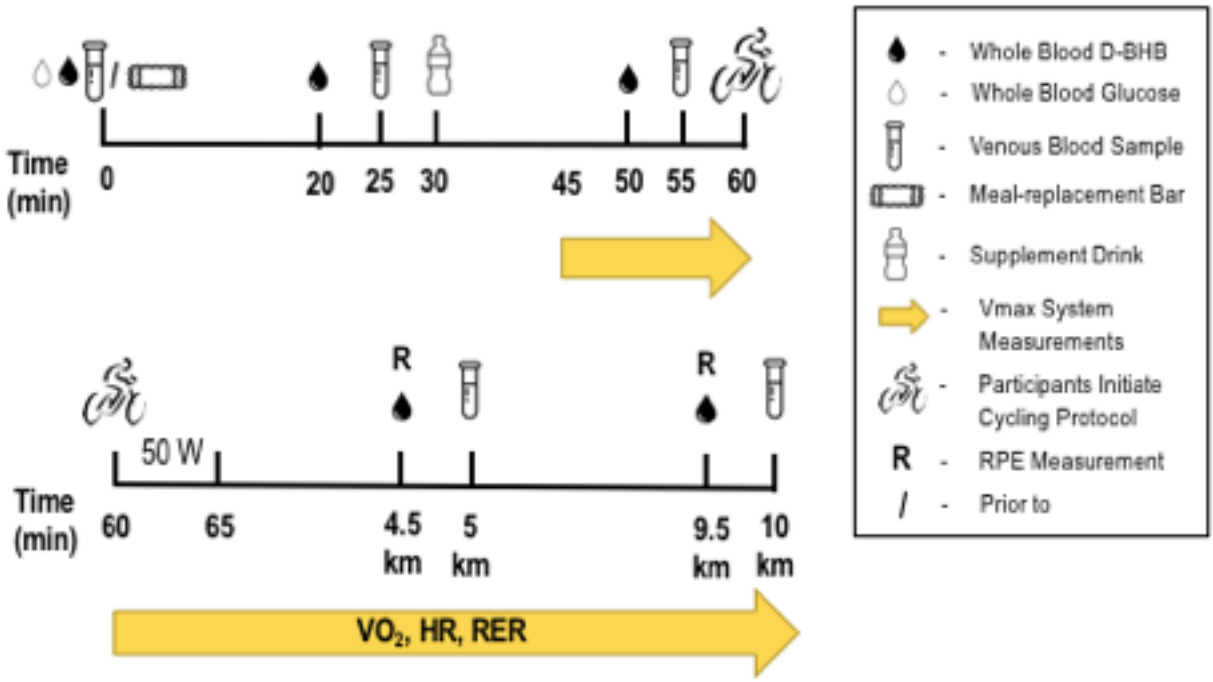

Figure 4. TT Laboratory Test Protocol. Participants arrived to the laboratory in a minimum 3-hour fasted state. Upon arrival, fingertip and venous blood samples were taken. A meal-replacement bar was consumed immediately following blood sampling and the supplement drink (KS or placebo) was ingested 30 mins later. Venous and fingertip blood measurements were taken again prior to supplement drink ingestion, prior to initiation of the time trial (TT) cycling protocol, and after a participant reached the 4.5 km and 9.5 km distances during the TT. To collect resting and exercise oxygen uptake (VO2), heart rate (HR) and respiratory exchange ratio (RER) data, Vmax system measurement collections began 15 mins prior to exercise initiation and were terminated after a participant reached the 10 km distance. Rate of perceived exhaustion (RPE) measurements were taken at the 4.5 km and 9.5 km distances of the TT.

Metabolic Rate Measurement

A Vmax system was used in this thesis to measure the metabolic rate of the participants. The student investigator set up the participant with a mouth piece attached to the flowmeter and headgear, and ensured the participant was wearing a nose clip. A customized test protocol was used on the Vmax software program that included the tabular collection of time, oxygen uptake (VO_2), respiratory exchange ratio (RER), and heart rate (HR). Metabolic measurements began with 15 minutes of resting measurements and commenced again once the submaximal workloads began. Vmax measurements were terminated once the participant had reached fatigue during the time to exhaustion protocol or had completed the 10-km distance during the time trial protocol. At this point, the student investigator removed the Vmax headgear and the participant continued cycling for a minimum of 5 minutes to ensure a sufficient cool-down time period was achieved.

In both exercise tests, the Vmax system collected data every 3 secs and various segments of the data were used for analysis. During the TT test, all Vmax data points from 0km to 10 km were averaged and used for analysis. During the TTE test, 10 minute segments of Vmax data points were used for analysis. From the 50% and 70% workloads, the data segment was taken from the 10 minutes following the initiation of each work load. The resting data segment was taken from the final 10 minutes of the resting work load. The fatigue data segment was taken from the final 10 minutes prior to the participants reaching fatigue. All 10-minute data segments were averaged to produce a single value for each participant at each work load. Averaged values from each participant were then used to compare differences between each phase of the study.

Heart Rate Measurement

An electrocardiogram was connected to the Vmax system to efficiently store and track heart rate values alongside the metabolic rate measurements. Heart rate measurements began

during the resting workload and continued until the participant reached fatigue. A three lead ECG placement was used to collect heart rate where the positive electrode was placed outside and just below the left nipple, the negative electrode placed on the center of the manubrium and the ground electrode placed outside and just below the right nipple. At the lab the student investigator used an alcohol swab to lightly scrub these areas on the participant before applying the disposable electrodes. Once the electrodes were placed and the correct leads were attached, the electrocardiogram was turned on and synced with the Vmax system.

Venous Blood Samples

Venous blood samples were collected using a BD Angiocath intravenous (IV) catheter, Discofix 3-way Stopcock and 5 cc/mL syringe. The lab technician began by wrapping an elastic tourniquet around the participant's arm. The IV catheter was then inserted into the cephalic vein within the cubital fossa. A strip of 3M Transpore tape was cut and used to hold the catheter in place. A 3-way stopcock was connected to the catheter and the tip of the stopcock and syringe were wiped with a PVP iodine swab. After attaching the syringe to the stopcock, the syringe was withdrawn to extract blood from the vein. The pre-determined volume of blood was transferred to an EDTA, SST or Fluoride Oxalate vacutainer.

The blood samples that were analyzed for serum insulin, cortisol, GH and NEFA were collected and transferred to an SST vacutainer to allow the blood to clot at room temperature for 30-60 mins. The two 5 ml SST vacutainers were then centrifuged to separate the blood serum. Multiple 500 μL aliquots of serum were transferred to each of the respective Eppendorf tubes using a pipette. All tubes were placed on ice until the student investigator transferred the samples to storage at -80 degrees Celsius until further analysis.

A 4 mL blood sample was obtained to be analyzed for glucagon. This sample was transferred from the syringe to an EDTA vacutainer and then inverted and centrifuged for 15 minutes at 1000 x g at a temperature of -8 degrees Celsius. A 500 μL aliquot of blood plasma for glucagon was added to a BD vacutainer and sealed. The samples were then immediately placed on ice within a portable cooler until transferred to storage at -20 degrees Celsius.

A 1.5 mL blood sample was obtained to analyze blood plasma lactate concentration. This sample was transferred to a Fluoride Oxalate Vacutainer and inverted and centrifuged immediately following the blood draw at a temperature of -8 degrees Celsius. A 500 μL aliquot of blood plasma was transferred to a labelled tube and stored at -80 degrees Celsius until further analysis.

Serum and Plasma Analysis

Serum concentrations of glucose, insulin, NEFA, cortisol and GH, as well as plasma concentrations of glucagon and lactate were measured and analyzed while participants completed the TTE cycling protocol. Conversely, during the TT cycling protocol, serum glucose and whole blood D-BHB were the only two metabolites measured and analyzed while participants completed the protocol.

Insulin and glucagon concentrations were measured by radioimmunoassay techniques (Human Insulin-Specific RIA and Glucagon RIA, EMD Millipore, Etobicoke, Ontario) using iodine-125 (^{125}I) as the labeled tracer antigen. During the assay procedures, a fixed concentration of ^{125}I-Insulin or ^{125}I-Glucagon was incubated with assay buffer and a constant dilution of anti-serum (Guinea Pig anti-Human Insulin Specific antibody or Guinea Pig anti-Glucagon serum) was added at a specific concentration in order to limit the number of binding sites on the antibody. When serum blood samples (unlabeled antigens) were combined with the labeled

tracer antigens, the unlabeled and labeled antigens competed for the limited and constant number of antibody binding sites, subsequently decreasing the amount of labeled antigen bound to antibody as the concentrations of unlabeled antigen increased. After both labeled tracer and antibody concentrations were added to unlabeled antigens, all samples were incubated for 20-24 hours. Precipitating reagent was then added to sample wells immediately following incubation to stimulate pellet formation. Samples were then centrifuged, decanted and counted on a Gamma Counter.

A colorimetric assay kit was used for analysis of serum NEFA (HR Series NEFA-HR(2)), Wako, Osaka, Japan). In this assay procedure, acyl-CoA synthetase, adenosine triphosphate, coenzyme A and 4-aminoantipyrine were added to serum blood samples to stimulate the production of acyl-CoA, adenosine monophosphate and pyro-phosphate. Acyl-CoA oxidase, 3-methyl-N-ethyl-N-(beta-hydroxyethyl)-aniline (MEHA) and peroxidase (POD) were then added to sample solutions to stimulate the production of hydrogen peroxide (H_2O_2). When POD is in the presence of H_2O_2, the oxidative condensation of MEHA with 4-aminoantipyrine is favoured, forming a purple colored end product. To determine the concentration of NEFA in each sample, optical density was measured at 550 nm, the absorption maximum of the purple colored end product.

An enzyme-immunoassay was used for cortisol and growth hormone (Cortisol ELISA Kit and Human Growth Hormone ELISA Kit, Abcam, Toronto, Ontario). In both assay procedures, plate wells arrived coated with anti-body (Anti-Cortisol IgG or Sheep anti-Human Growth Hormone). Antibody-enzyme conjugate solutions (Cortisol-horseradish peroxidase or mouse monoclonal anti-Human Growth Hormone antibody and horseradish peroxidase) were then added to standard, control and sample wells. Plate wells were then covered and incubated for 45-

60 min. Wells were then washed three times with diluted washing solution and tetramethylbenzidine reagent was added to stimulate the development of a blue colour in plate wells. Color development was stopped using 1N hydrochloric acid, changing the plate well colours from blue to yellow. The colour intensity of the test sample was directly proportional to the concentration of unlabeled cortisol or growth hormone in each sample. Absorbance of each well was measured spectrophotmetrically at 450 nm.

Spectrophotometric assays were used for analysis of plasma concentrations of lactate and glucose. The plasma lactate assay procedure began with preparing the reagent solution via mixing hydrazine, glycine and nicotinamide adenine dinucleotide. The reagent was then diluted and added to all standard, control and sample tubes, which were immediately vortexed. Diluted LDH was added to all tubes, which were then vortexed and incubated in the dark for 120 min. Tubes were then read on a fluorometer using an excitation wavelength of 530 nm. To initiate the glucose assay procedure, PGO enzyme preparation and a-Dianisidine Solution were mixed and added to standards, controls and plasma samples. Tubes were then centrifuged and incubated for 30 min in a water bath set to 37 degrees Celsius. Incubation stimulated the production of a yellow colour in tubes, which were subsequently read on a spectrophotometer at 450 nm.

Statistics

Collected data are reported as the mean $\pm$ SEM. A paired t-test was used to determine if there was a significant difference in time to exhaustion and distance traveled between both energy supplements for all participants. A two-way repeated measures ANOVA was used to determine if there was a significant interaction effect of time and condition between metabolic measures of HR, RER, VO_2, and blood measures of insulin, glucagon, cortisol, catecholamines, βHB, lactate and FFA. A Tukey's HSD Post-Hoc test was used when statistical significance was

found. Results with $P < 0.05$ were considered to be significant.

Results

Participant Experiences

Eight varsity athletes commenced this study, however, one participant chose not to complete the second phase of the study due to adverse physical reactions to the insertion of the venous catheter prior to the exercise tests. Consequently, data from this participant have been removed from analysis.

Participants were randomly assigned a supplement for the first phase of the study, with 4 participants consuming KS during their first phase of the study and 3 participants consuming KS during their second phase. From the 7 participants that completed the study, 4 participants correctly nominated the trial in which they received the ketone supplement while 3 participants guessed incorrectly. The eighth participant (who chose not to complete the exercise tests during phase 2 of the study) did complete the 5-day supplementation protocol in both phases of the study. When asked which supplement was taken during each supplementation phase, this participant also guessed incorrectly. Therefore, using all 8 participants, a correct guess percentage of 50% would indicate that the ketone supplement was successfully masked. Additionally, only 1 participant reported gastrointestinal discomfort after ingesting the ketone supplement, describing a state of mild stomach discomfort for the 30 min following ingestion of the supplement, with symptoms subsiding shortly thereafter.

Dietary Intake

During laboratory testing on day 1,5,6 and 8, all participants were asked to consume the same meals for the 24 hours prior to exercise testing during each phase of the study. Twenty-four hours prior to each test date, the student investigator contacted participants to remind them of this dietary protocol and the following morning, 24-hour dietary intake was confirmed and

recorded between the participants and student investigator. Based on the dietary records received from all participants during the entire course of the study, all participants consumed the exact same meals within the 24-hours prior to laboratory testing on day 1,5,6 and 8, during both phases of the study.

Baseline Characteristics and Participant Performances

Participants recruited for this study were on average 25.6 $\pm$ 0.3 years of age with an average height of 182 $\pm$ 2.6 cm and average weight of 87.7 $\pm$ 3.4 kg. During the initial step-wise, maximal exercise test, participants had an average VO_2max of 52.9 $\pm$ 3.1 mL/kg/min. From VO_2max testing, the average stationary bike workloads among all participants during the TTE protocol were calculated to be 163.6 $\pm$ 2.8 Watts and 201.4 $\pm$ 3.7 Watts for the 50% and 70% VO_2max stages of the test, respectively. During the TT test, all participants rode at a consistent resistance of 2.5 kg.

Fingertip Blood Sampling (Day 1-5)

Participants arrived to the laboratory for fingertip blood sampling on day 1 and 5 during both phases of the study. After completing a minimum 3-hour fast, whole blood measurements of glucose (Figure 5A) and D-BHB (Figure 5B) were taken. Average fasting glucose concentration was not significantly ($p = 0.813$) different between day 1 and day 5 for either supplementation phase of the study or between placebo and KS supplementation. Within each supplementation phase, there were no statistical differences in blood D-BHB concentrations between day 1 and day 5, therefore, day 1 and day 5 data were averaged to compare differences between supplementation phases. Average blood D-BHB concentrations measured upon arrival (0 min) and following meal-replacement bar ingestion (30 min) were not statistically different between placebo and KS phases of the study; however, KS significantly elevated ($p < 0.001$) blood D-

BHB concentrations 30 min following supplement ingestion, with concentrations remaining significantly elevated ($p < 0.002$) until the last measurement at 120 min (Fig. 5B). During this period, blood D-BHB concentrations were significantly elevated ($p < 0.001$) compared to the placebo.

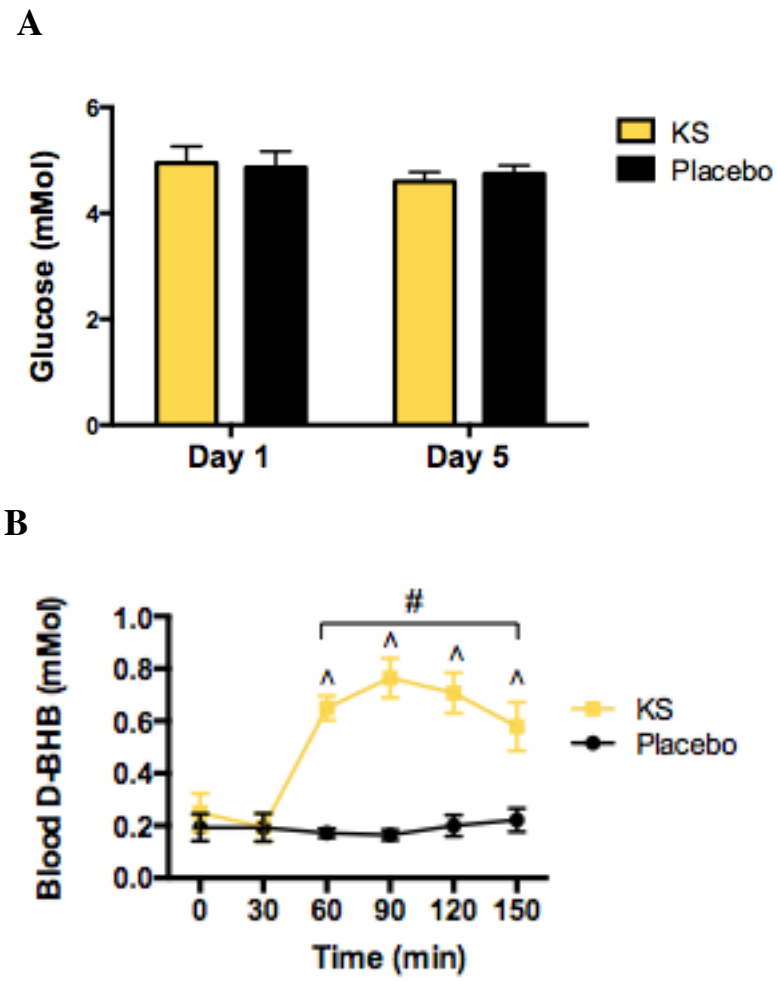

Figure 5. Whole blood measurements of glucose and D-BHB from fingertip blood sampling (Day 1-5). Fasting glucose concentration comparisons between day 1 and day 5 of KS and placebo supplementation (**A**). Average whole blood D-BHB concentrations between KS and placebo at baseline and for 2 hrs following supplementation (**B**). All measurements were taken immediately following arrival to the laboratory. A meal replacement bar was ingested immediately following fasting measurements (0 min) and supplements were then ingested immediately following measurements at the 30 min time point. # indicates KS significantly elevated concentrations compared to placebo ($p < 0.003$), ^ indicates concentrations were significantly elevated compared to baseline measures ($p < 0.003$).

Performance, metabolite and hormone responses during the TTE protocol (Day 6).

Ketone salt supplementation significantly increased ($p < 0.04$) time to fatigue during the 70% workload of the TTE stationary bike protocol compared to placebo supplementation (Fig. 6A). This corresponded with a significantly decreased ($p < 0.05$) rate of perceived exertion during the KS phase compared to the placebo phase (Fig. 6B). During rest and throughout the entire cycling protocol, HR was not significantly different between KS and placebo (Fig. 6C). Compared with placebo, RER was significantly reduced ($p < 0.05$) at rest but not during exercise, following KS ingestion (Fig. 6D). As expected, all exercise measurements of RER during the KS and placebo phase were significantly elevated compared to resting values. During the 70% VO_2max measurement time period, VO_2 was significantly reduced ($p < 0.05$) during the KS phase compared to placebo; however, VO_2 at rest, 50% VO_2max and fatigue was not statistically different between KS and placebo (Fig. 6E).

There were no statistical differences in fasting serum glucose between the KS and placebo phases of the study, and despite a significantly elevated ($p < 0.05$) serum glucose concentration during the pre-supplement ingestion time point in the KS phase, all other time points during the TTE laboratory test day were not statistically different between study phases (Fig. 7A). As expected, blood D-BHB concentrations were not different between KS and placebo during fasting and pre-supplement time points but were elevated above baseline ($p < 0.001$) following KS supplementation and were significantly greater ($p < 0.02$) compared to placebo from the pre-exercise to fatigue time point of the TTE protocol (Fig. 7B).

Using a cross-over, repeated measures design, participants were advised to consume the exact diet and follow the exact activity patterns in phase two of the study as they did during phase one of the study. It was assumed that fasting and pre-supplement ingestion values of

plasma lactate, NEFA, insulin, glucagon. cortisol and growth hormone would be the same between both phases, thus assays were only completed at the pre-exercise (post-supplement ingestion), 50% VO_2max and fatigue time points of the TTE protocol. Plasma lactate concentration increased ($p < 0.05$) incrementally from pre-exercise to fatigue but there were no statistical differences between KS and placebo (Fig. 8A). Compared with placebo, plasma NEFAs were not different during pre-exercise or 50% VO_2max measurement time points but were significantly elevated ($p < 0.03$) at fatigue (Fig. 8B) with KS supplementation. During the 50% VO_2max work rate, serum NEFA concentrations were significantly reduced ($p < 0.04$) in both study phases compared to pre-exercise measurements, however, concentrations at fatigue were significantly elevated ($p < 0.05$) in both phases compared to pre-exercise measurements.

Insulin was significantly increased in the KS phase versus placebo during the pre-exercise ($p < 0.05$) and 50% VO_2max work rates ($p < 0.04$), but no differences were found between the KS and placebo phases at fatigue (Fig. 9A). Serum insulin concentrations during exercise in both phases were significantly reduced ($p < 0.04$) compared to pre-exercise measures, and concentrations at fatigue were significantly reduced ($p < 0.03$) compared to the 50% VO_2max time point. In contrast to insulin, plasma glucagon was significantly reduced ($p < 0.05$) in the KS phase compared with placebo at both pre-exercise and 50% VO_2max measurement time points, but no differences were found at fatigue between phases (Fig. 9B). Plasma glucagon concentrations were significantly elevated during exercise in both phases compared to pre-exercise measures, while concentrations at fatigue were significantly increased ($p < 0.04$) compared to the 50% VO_2max work rate. No statistical differences were found in serum cortisol (Fig. 10A) or serum growth hormone (Fig. 10B) between KS and placebo. As expected, concentrations of both hormones were significantly elevated ($p < 0.05$) during fatigue compared

to pre-exercise measures, while significant elevations in growth hormone ($p < 0.03$) but not cortisol were also observed during the 50% VO_2max work rate compared to pre-exercise measures.

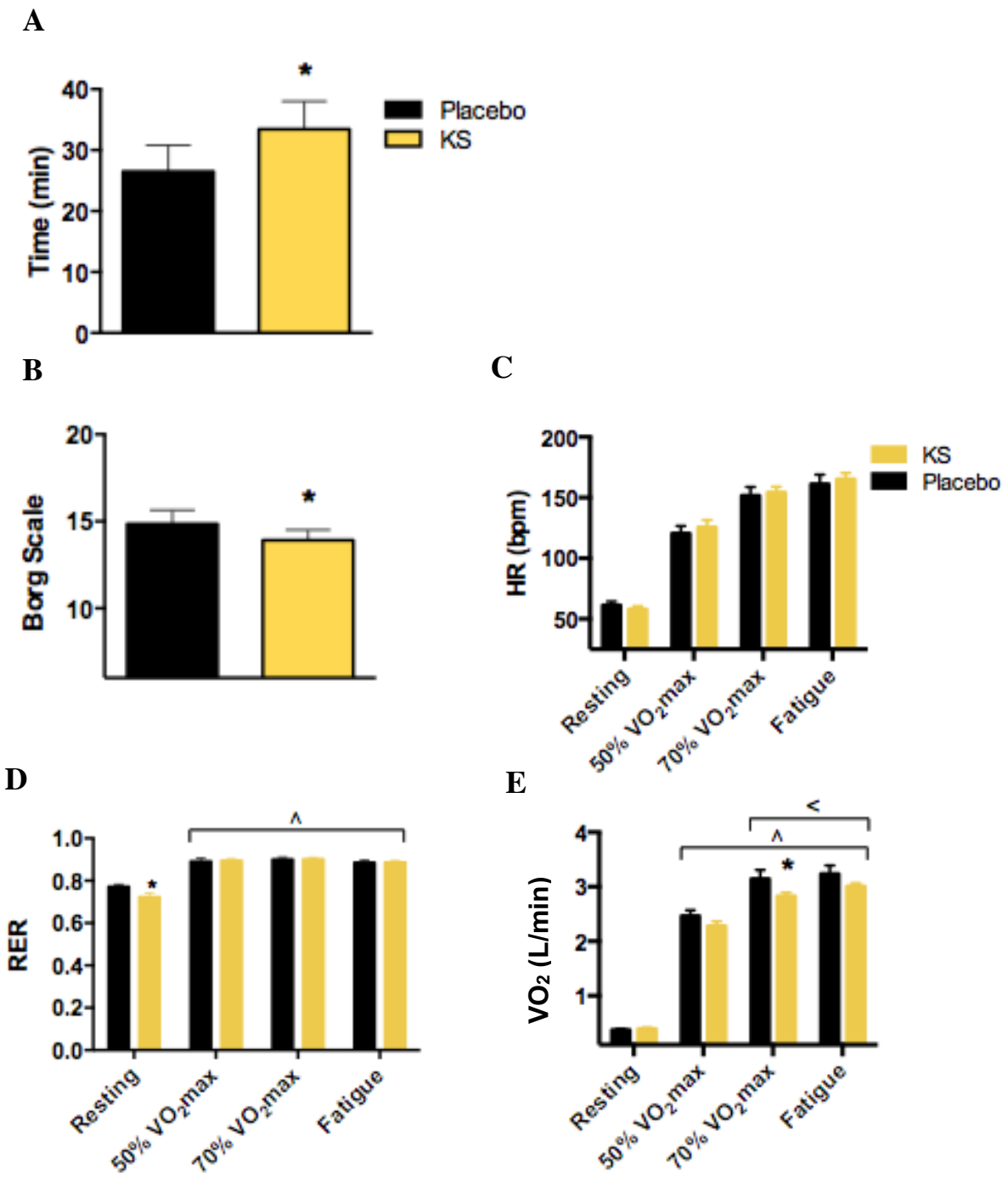

Figure 6. Performance measurements during the TTE protocol (Day 6). (**A**) Time to fatigue during 70% VO_2max workload. (**B**) Average rate of perceived exhaustion (RPE) during the 50% and 70% VO_2max workloads of the TTE protocol. Vmax system measurements of (**C**) heart rate, (**D**) respiratory exchange ratio and (**E**) oxygen uptake during the various stages of the TTE protocol. * indicates that the KS condition was significantly different from the placebo condition ($p < 0.05$), ^ indicates a time point that is significantly elevated compared to baseline ($p < 0.05$), < indicates a time-point that is significantly elevated compared to 50% VO_2max ($p < 0.04$).

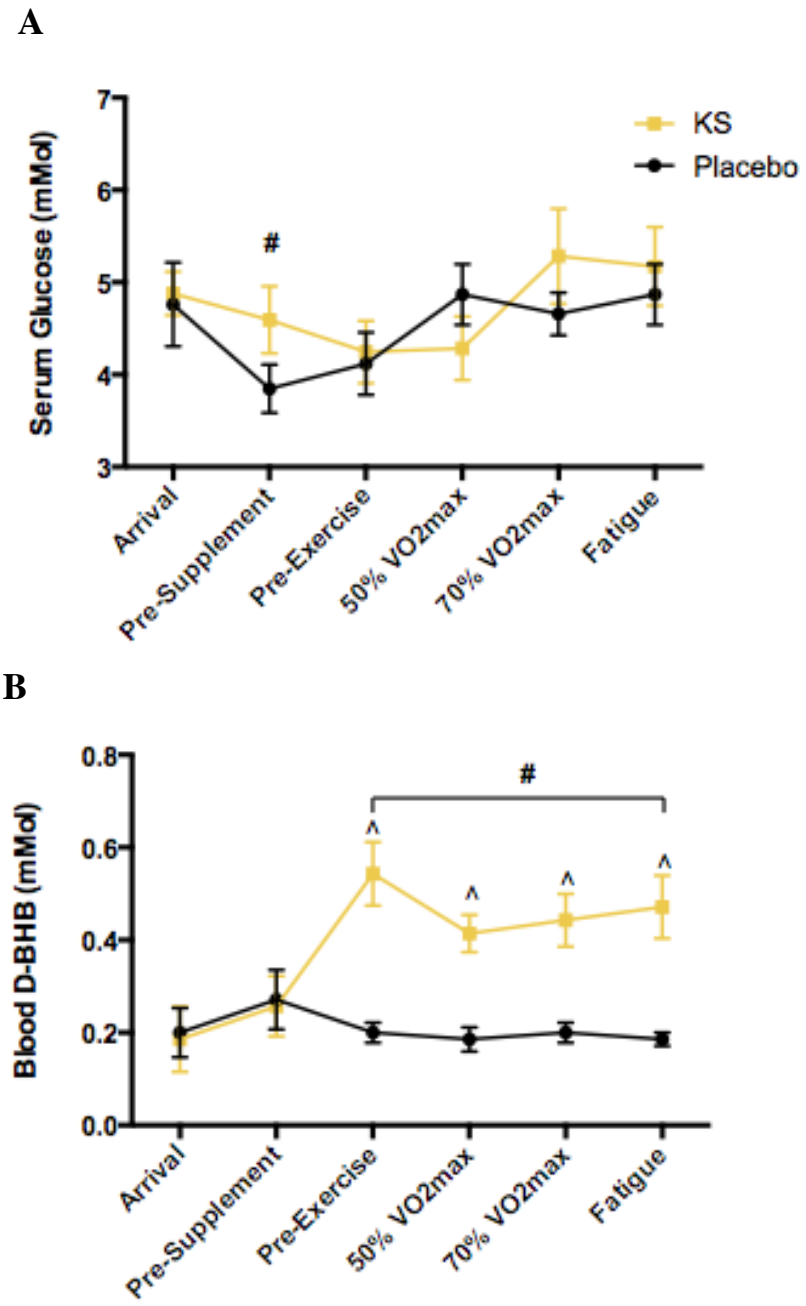

Figure 7. Glucose and D-BHB concentration responses during the TTE protocol (Day 6). (**A**) Serum glucose concentrations and (**B**) whole blood D-BHB concentrations measured from a portable ketone meter device during various time points prior to and after completing the TTE stationary bike test protocol. # indicates KSs significantly elevated concentrations compared to placebo ($p < 0.05$), and ^ indicates concentrations are significantly elevated from baseline ($p < 0.04$).

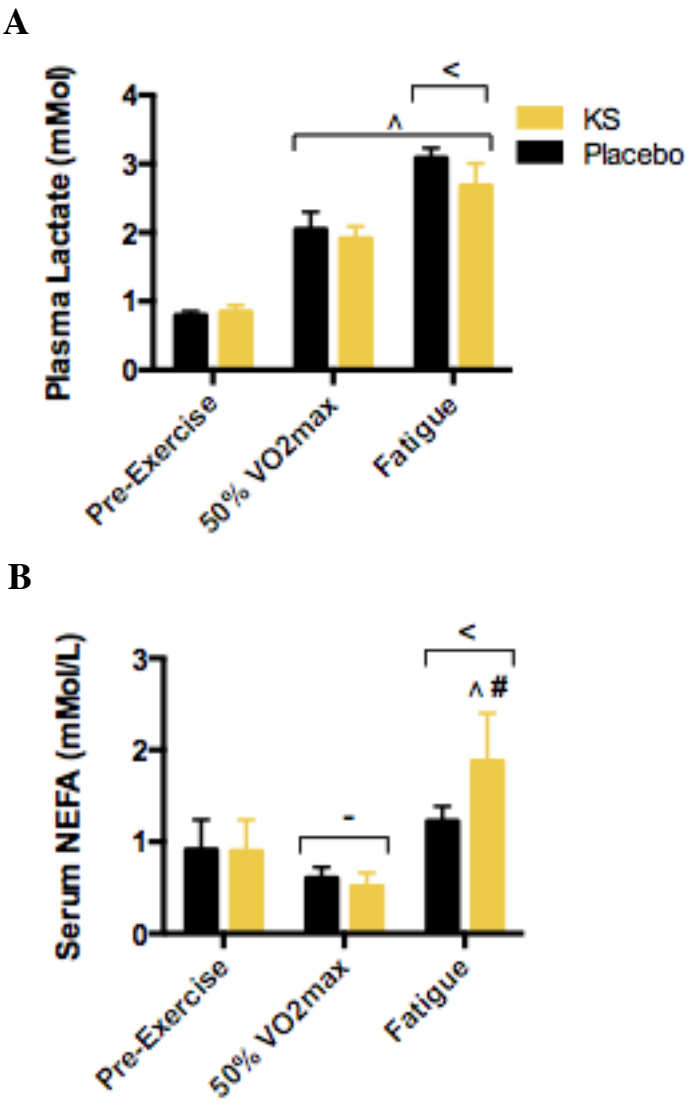

Figure 8. Lactate and NEFA concentration responses during the TTE protocol (Day 6). Plasma concentrations of (**A**) lactate and (**B**) serum concentrations of non-esterified fatty acids during various time points prior to and after completing the TTE stationary bike test protocol. ^ indicates a time point that is significantly elevated compared to baseline ($p < 0.05$), < indicates a time point that is significantly elevated compared to 50% VO_2max ($p < 0.04$), - indicates a time-point that is significantly reduced compared to baseline ($p < 0.05$), and # indicates KSs significantly elevated concentrations compared to placebo ($p < 0.05$).

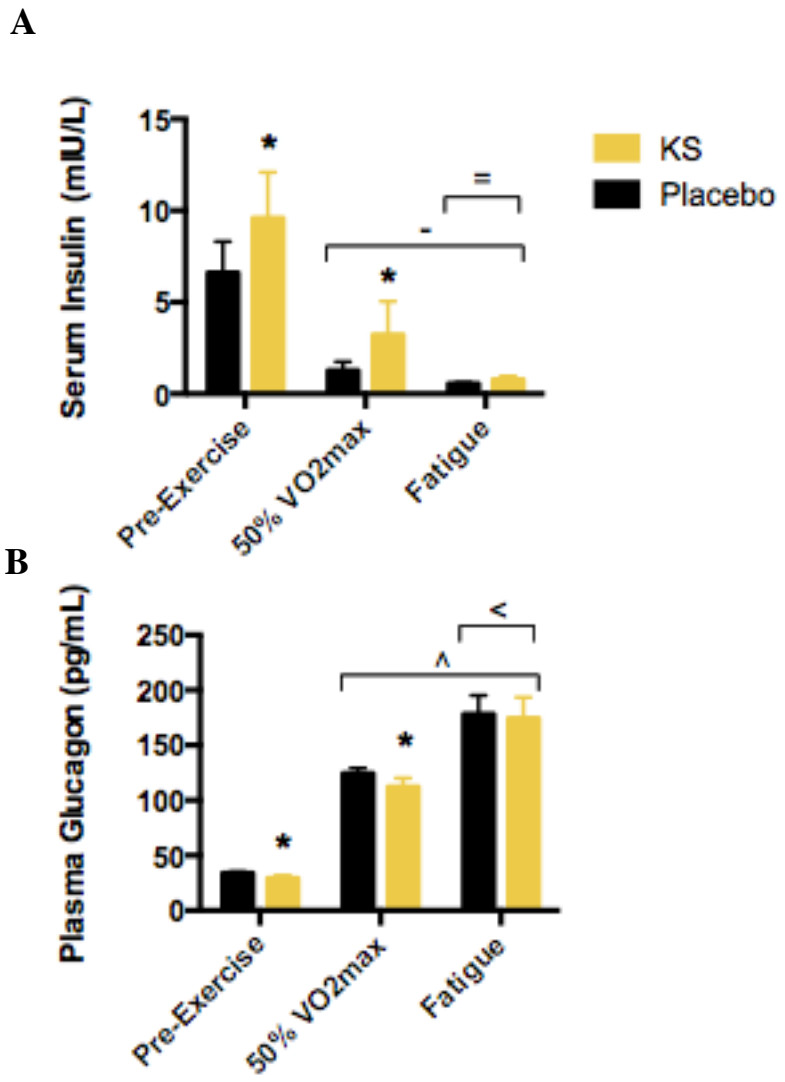

Figure 9. Insulin and glucagon concentration responses during the TTE protocol (Day 6). Plasma concentrations of (**A**) insulin and (**B**) serum concentrations of glucagon during various time points prior to and after completing the TTE stationary bike test protocol. * indicates the KS condition was significantly different compared to the placebo condition ($p < 0.05$), - indicates a time point that is significantly reduced compared to baseline ($p < 0.05$), = indicates a time point that is significantly reduced compared to 50% VO_2max ($p < 0.05$), ^ indicates a time point that is significantly elevated compared to baseline ($p < 0.04$), < indicates a time point that is significantly elevated compared to 50% VO_2max ($p < 0.04$).

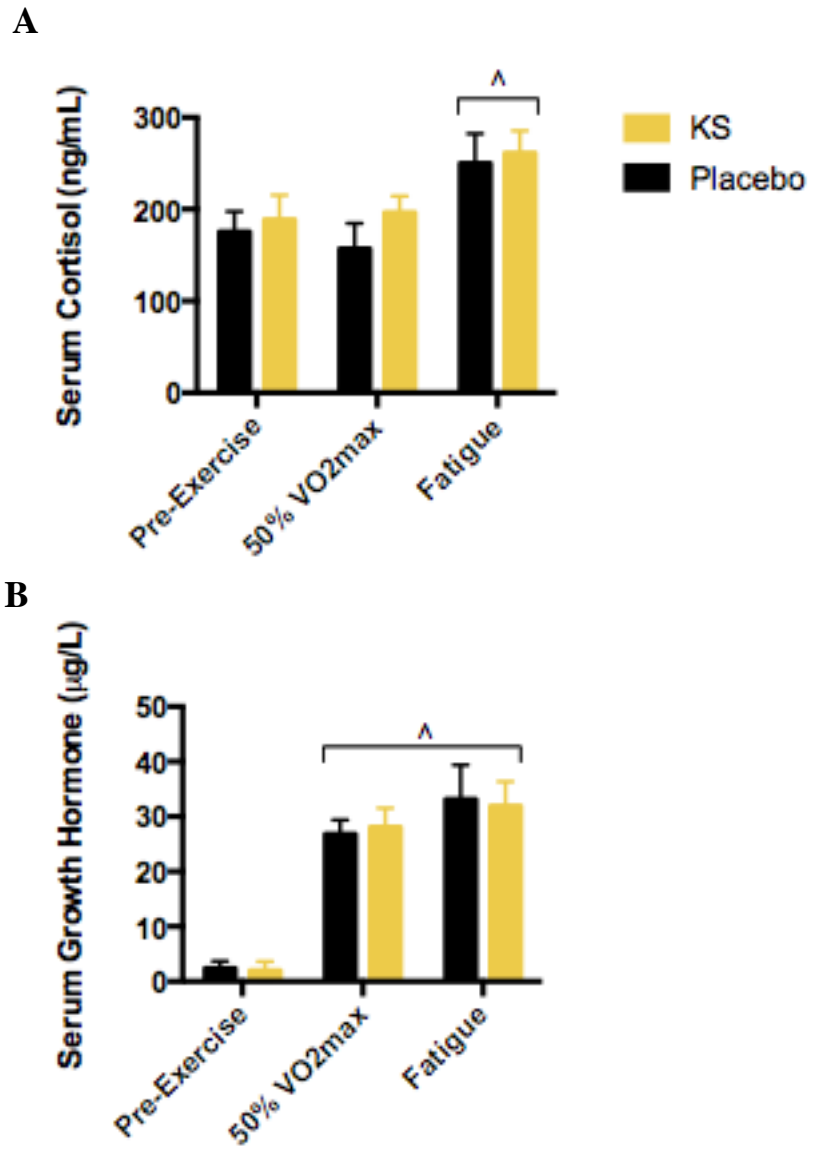

Figure 10. Hormone response during the TTE protocol (Day 6). Venous blood concentrations of (**A**) cortisol and (**B**) growth hormone during various time points prior to and after completing the TTE stationary bike test protocol. ^ indicates a time point is significantly elevated compared to baseline ($p < 0.04$).

Performance, glucose and D-BHB responses during the TT protocol (Day 8).

Performance measurements for the TT protocol are shown in Fig. 11. No differences between KS and placebo were observed in total time needed to complete the 10-km duration (Fig. 11A), RPE (Fig. 11B) or HR (Fig. 11C). Compared with placebo, RER was significantly reduced ($p < 0.003$) at rest but not during the TT, following KS ingestion (Fig. 11D). Surprisingly, resting VO_2 was significantly elevated ($p < 0.003$) following KS ingestion compared with placebo; however, VO_2 during the TT was not statistically different between KS and placebo (Fig. 11E).

Venous blood glucose concentrations and whole blood D-BHB concentrations are shown in Fig. 12. There were no statistical differences in serum glucose concentrations between KS and placebo at an point during rest or exercise nor between rest and exercise for either KS or placebo (Fig. 12A). Blood D-BHB concentrations during fasting and pre-supplement time points were not different between KS and placebo; however, blood D-BHB concentrations were significantly greater ($p < 0.002$) in the KS phase compared to the placebo from the pre-exercise to 9.5 km time point of the TT protocol (Fig. 12B). At these three time points, concentrations during the KS phase were also significantly elevated ($p < 0.002$) compared to baseline values.

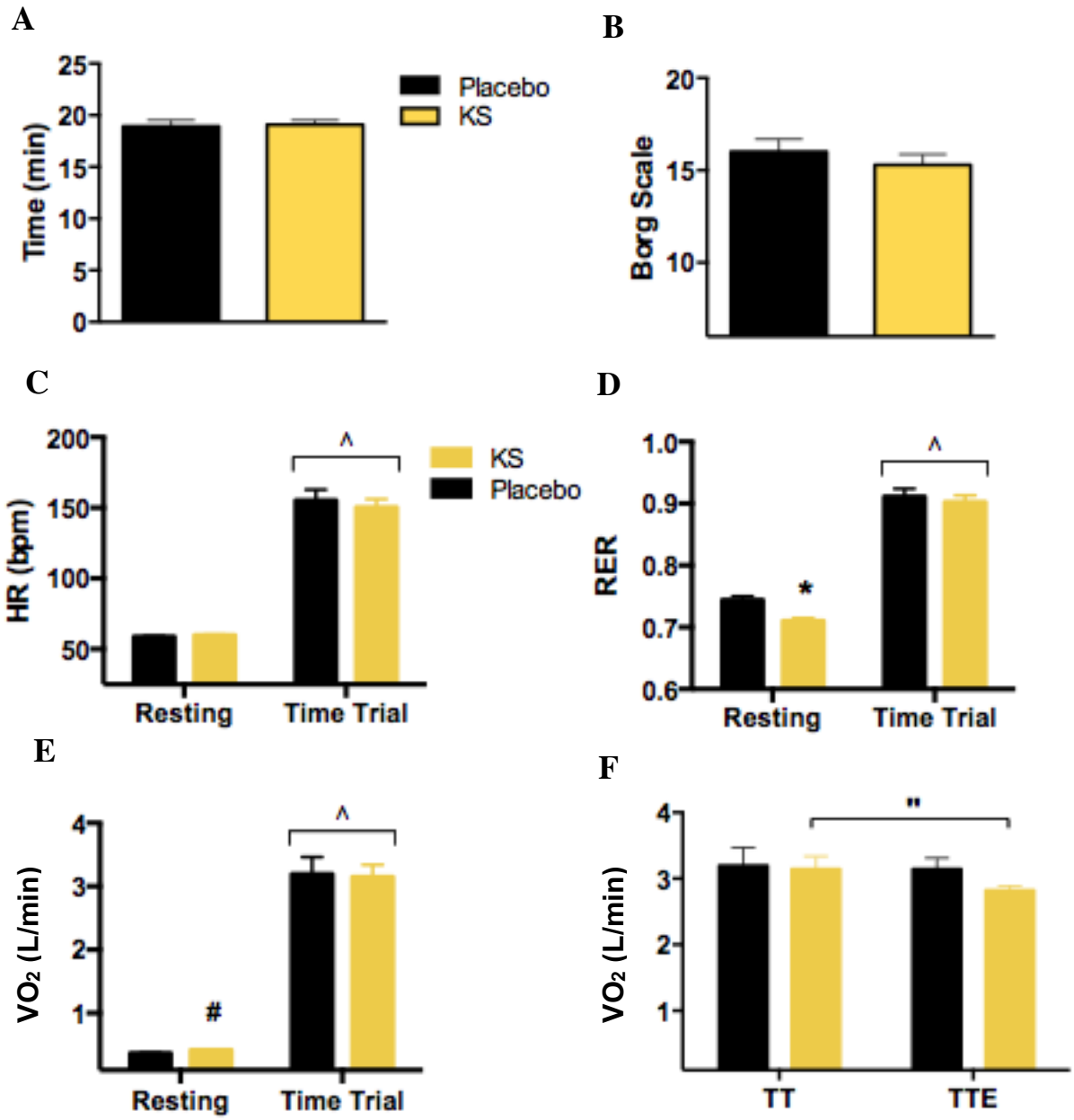

Figure 11. Performance measurements during the TT protocol (Day 8). (**A**) Time to complete the 10-km stationary bike TT. (**B**) Average rate of perceived exhaustion during the TT. Vmax system measurements of (**C**) heart rate, (**D**) respiratory exchange ratio and (**E**) oxygen uptake during the TT. Oxygen uptake during exercise in the TT is compared with oxygen uptake during the 70% VO2max measurement time point of the TTE (**F**). * indicates KSs significantly reduced measurement parameters compared to placebo ($p < 0.05$), ^ indicates a time point significantly elevated compared to baseline, # indicates KSs significantly elevated measurement parameters compared to placebo ($p < 0.05$), and " indicates a significant difference between exercise tests during KS supplementation ($p < 0.03$).

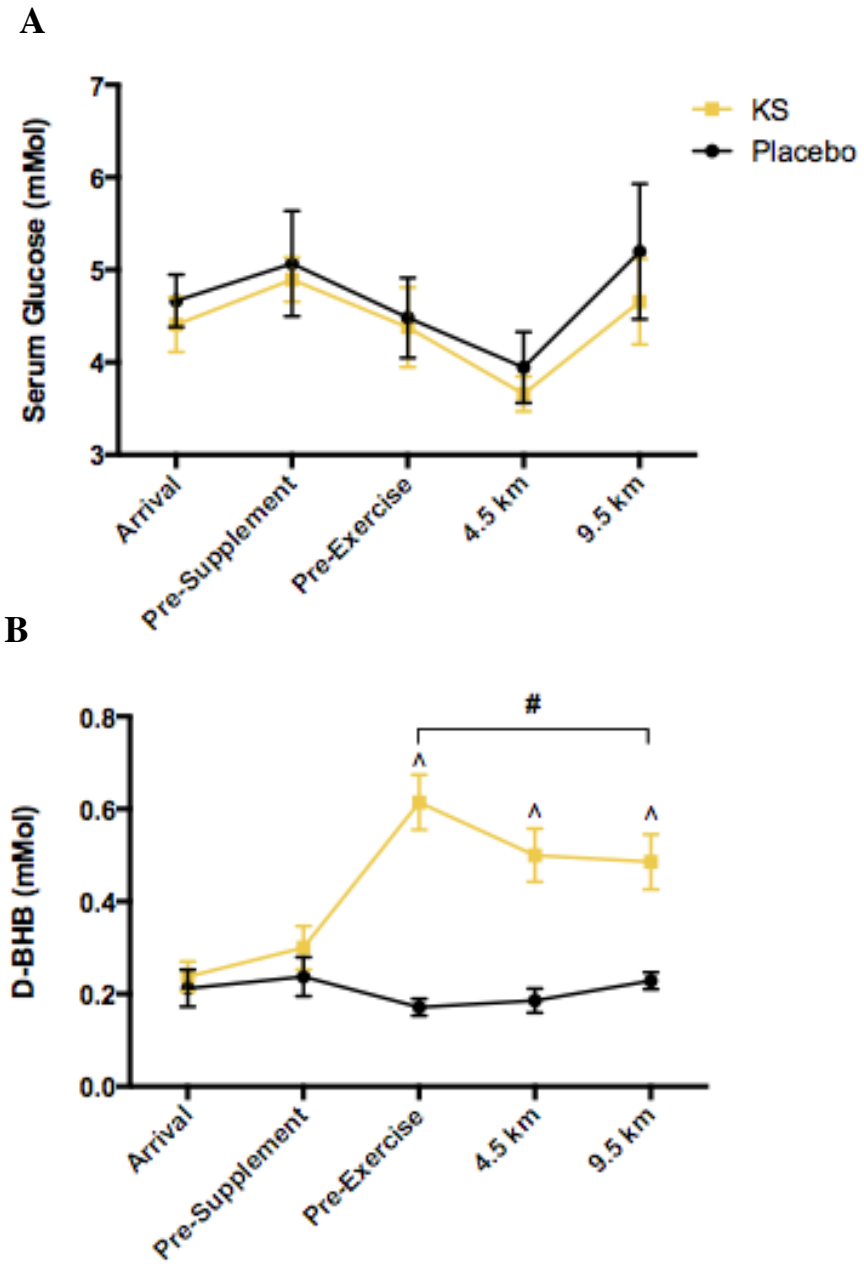

Figure 12. Glucose and D-BHB response during the TT protocol (Day 6). (**A**) Serum glucose concentrations and (**B**) whole blood D-BHB concentrations measured from a portable ketone meter device during various time points prior to and after completing the TT stationary bike test protocol. # indicates KSs significantly elevated concentrations compared to placebo ($p < 0.002$), ^ indicates concentrations are significantly elevated compared to baseline ($p < 0.002$).

Discussion

The aim of this thesis study was to characterize the effects of BHB salt and MCT ingestion on the pharmacokinetic and pharmacodynamic whole blood D-BHB response at rest, as well as the effects of twice-daily consumption of a BHB plus MCT supplement for 5-8 consecutive days on exercise performance, metabolic efficiency and the endocrine response to exercise. Notably, a single-dose of BHB salts plus MCTs significantly elevated blood D-BHB concentrations above 0.1 mM within 30 mins of consumption, with concentrations remaining significantly elevated above baseline for a minimum of 120 min. When BHB salts plus MCTs were consumed twice-daily for 6 days prior to completing a TTE cycling protocol, metabolic efficiency and time to fatigue were significantly increased, while rate of perceived exhaustion was decreased compared to placebo ingestion. When BHB plus MCTs were consumed twice-daily for 8 days prior to a TT cycling protocol, no differences were found in exercise performance or rate of perceived exhaustion compared to placebo ingestion. During the TTE, insulin was elevated and glucagon was reduced during rest and at the 50% VO_2max measurement time point, though no differences were seen in insulin, glucagon, lactate, cortisol and GH at fatigue. Based on these measurements alone, BHB and MCT supplementation was shown to blunt the endocrine response at rest and during 50% VO_2max exercise compared to placebo, however, no differences were seen in the endocrine response to exercise at fatigue.

With reference to whole blood D-BHB, the moderate BHB salt dose used within this study of 0.3 g/kg body mass and a MCT dose of 0.18 g/kg body mass, increased average blood D-BHB concentrations to 0.65 $\pm$ 0.04 mM, peaking at 0.76 $\pm$ 0.07 mM 1 hour following supplement ingestion. This modest elevation in blood D-BHB concentration following an average BHB dose of ~26 g is consistent with previous studies that achieved concentrations

ranging from 0.4-1.0 mM following BHB doses of ~21 g to 27 g (O'Malley et al., 2017; Rodger et al., 2017; Short, 2017; Stubbs et al., 2017; Evans et al., 2018). However, the BHB salt plus MCT supplement failed to elevate and sustain blood D-BHB concentrations to ~1-2 mM, the estimated concentration of skeletal muscle D-BHB saturation (Balasse and Fery, 1989; Evans et al., 2016; Stubbs et al., 2017). Furthermore, the peak blood D-BHB levels observed here are much lower relative to blood D-BHB concentrations reported following a single bolus of KE supplements (i.e. ~2.0 – 6 mM) (Cox et al., 2016).

Commercially available BHB salt supplements often contain a racemic mixture of BHB isoforms, increasing blood concentrations of both D-BHB and L-BHB (Stubbs et al., 2017). Surprisingly, when a ketone diester supplement is ingested, blood D-BHB concentrations are moderately elevated to similar ranges as seen with BHB salts during exercise (~0.3 – 0.4 mM) (Leckey et al., 2017). Upon further investigation, the ketone diester 1,3-butanedial acetoacetate contains a racemic mixture of D-BHB and L-BHB, similar to that found in BHB salts. It is unknown whether a BHB salt supplement containing solely D-BHB would increase blood D-BHB concentrations similar to a ketone monoester supplement (Evans et al., 2017, Cox et al., 2016).

Additionally, moderate elevations in blood D-BHB concentrations within this present study may have been a consequence of consuming the KS in a fed-state, as participants consumed 22 g of CHO 30 minutes prior to KS ingestion. As reported by Cox et al., (2016) the co-ingestion of a ketone monoester with dextrose increased peak D-BHB concentrations to ~3.2 mM compared to ~ 6.2 mM when the ketone monoester was ingested alone. Despite the potential effect of CHO on D-BHB concentrations, 5 day supplementation of a BHB salt plus MCT supplement did not significantly alter blood D-BHB concentrations compared to day 1 of the KS

phase. Although no blood D-BHB concentration changes were observed, it is important to note that keto-adaptive responses may still have occurred, particularly through increasing intestinal absorption and peripheral tissue uptake of D-BHB (Phinney, 2004; Volek and Phinney, 2015; McSwiney et al., 2018). Hence, if both were proportionally increased over the 5-day supplementation protocol, blood D-BHB concentrations would stay consistent with concentrations on day 1 despite increasing KB utilization at rest (Evans et al., 2017).

BHB salt and MCT supplementation increases time to fatigue during a TTE cycling test

This is the first known study to report that 6 consecutive days of KS plus MCT supplementation increases time to fatigue during a 2-stage sub-maximal steady state (50 and 70% VO_2max) TTE cycling protocol by ~20% in male varsity athletes. During this study, KS plus MCT supplementation was also shown to alter mechanical efficiency, as noted by a significantly reduced VO_2 during the 70% VO_2max measurement time point compared to placebo supplementation. Both of these findings were in accordance with our hypotheses, as KS plus MCT supplementation was expected to increase mechanical efficiency and exercise performance compared to placebo supplementation based on previous research showing increased cardiac output and ATP production, and reduced VO_2 with rat hearts perfused with D-BHB, AcAc and glucose compared to glucose perfusion alone (Veech et al., 2001). The exact mechanism by which KS plus MCTs improved mechanical efficiency during the TTE cycling test is not fully understood, though several factors may have been at play. Firstly, the inherent efficiency advantage of KB over CHO as an oxidative fuel source must be noted (Sato et al., 1995; Veech et al., 2004). Veech et al., (2004) speculate that the increase in mechanical efficiency was most likely due to the greater ΔG of ATP hydrolysis produced within ATP synthesized during terminal oxidation of KB. Specifically, during ATP synthesis, the ΔG of ATP

hydrolysis potential is stored within the third phosphate bond of ATP, therefore, hydrolysis of ATP synthesized during terminal oxidation of KB would yield a greater amount of energy then ATP synthesized during terminal oxidation of glucose. Put simply, less ATP would be needed to fuel constant load exercise when KB and glucose are terminally oxidized versus glucose oxidation alone. However, it is important to note that oxygen uptake was not statistically different during the resting, 50% VO_2max and fatigue measurement time periods. It is not fully understood why mechanical efficiency was not altered during these measurement time points, though it is speculated that terminal oxidation of KB was most likely limited at these times.

To further explore the performance benefits of increased ATP synthesis and decreased ATP hydrolysis during a TTE test, the factors affecting fatigue during this type of exercise protocol must be addressed. When constant load exercise is maintained until fatigue, it is well established that fatigue is achieved by either central or peripheral fatigue mechanisms, though peripheral fatigue mechanisms are most common (for review see Allen et al., 2008). Within skeletal muscle, peripheral fatigue is caused by various metabolic and/or non-metabolic fatigue factors, which negatively alter excitation and contraction processes. During metabolic fatigue, energy supply is reduced and/or the concentration of metabolic by-products significantly increases, negatively altering excitation and contraction processes. During non-metabolic fatigue, structural proteins and membranes involved in excitation and contraction processes are damaged by factors such as free radicals and proteolytic enzymes, decreasing the ability for these proteins and membranes to function. Based on the principle that participants exercised to exhaustion during the TTE cycling test in this study, KB and glucose oxidation would be expected to reduce both metabolic and non-metabolic factors of fatigue compared to glucose oxidation alone and therefore increase time to exhaustion. Respectively, when ATP synthesis is increased and ATP

hydrolysis is reduced following KB and glucose oxidation, energy supply would increase while the build-up of metabolites such as adenosine diphosphate, adenosine monophosphate and inorganic phosphate would decrease. Additionally, terminal oxidation of KB within the ETC is known to reduce the production of free radicals such as superoxide and hydrogen peroxide and increase the production of the antioxidant glutathione (Veech et al., 2001; Veech, 2004). Therefore, non-metabolic fatigue may be reduced following KB and glucose oxidation compared to glucose oxidation alone.

Interestingly, though KB oxidation may have altered metabolic and non-metabolic peripheral fatigue factors, the elevation of blood KB concentrations may have also positively altered mechanisms of central fatigue. It is well established that central fatigue is achieved when the number of functioning motor units required to maintain work output is decreased and/or the stimulation frequency of these motor units is decreased (for review see Gandevia, 2001). When neural input to skeletal muscle motor units is impaired, psychological and/or physical mechanisms such as reflex drive and neuromuscular transmission may be at fault. With respect to psychological impairments, KB oxidation may alter the psyche and motivation of an individual, as average RPE during the TTE protocol was significantly lower during the KS plus MCT supplementation compared to placebo. Whether this is a direct effect of KB oxidation or an indirect effect of D-BHB signaling is yet to be fully understood. Regardless of the mechanisms, exogenous ketones may positively alter the psychological experience of exercise, as Leckey et al. (2017) stated that multiple world-class cyclists experienced a "centrally-driven" enhanced mental state when exogenous ketones were ingested prior to a laboratory cycling test.

Notably, psychological and exercise performance improvements following an acute dose of KS plus MCT have yet to be reported. Therefore, it is likely that the 6-day supplementation

protocol or the addition of MCTs to the KS supplement may have played an important role in improving exercise performance during the TTE in this study. In the case of the former, twice-daily supplementation of BHB salts plus MCT may have stimulated keto-adaptive processes, increasing skeletal muscle KB utilization during exercise. Based on the principles discussed above, increased skeletal muscle KB utilization would be expected to positively alter peripheral factors of fatigue, thus increasing subsequent exercise during a fatigue protocol (Evans et al., 2017). In the case of the latter, the addition of MCTs into the BHB supplement formula may have also improved performance, as MCT consumption alongside CHO has been shown to decrease total time to complete a 40-km cycling time trial compared to CHO ingestion alone (Van Zyl et al., 1996). Additionally, MCTs are known to stimulate acute hyperketonemia, hence, the addition of MCTs to the supplement formula may have also played a minor role in promoting greater KB utilization in skeletal muscle (D'Agostino et al., 2017).

In order to determine the effects of KB plus MCT supplementation on the endocrine or counter-regulatory response to exercise, blood concentrations of glucose, insulin, glucagon, cortisol and growth hormone were measured. At present, this is the only known study to report the effects of KS plus MCT supplementation on glucose, lactate, glucagon, insulin, cortisol and GH within human participants. It was hypothesized that KS plus MCT ingestion would alter the hierarchy of substrate competition for respiration during rest and exercise, specifically increasing KB utilization and reducing CHO utilization in skeletal muscle. Based on the premise that the counter-regulatory response to exercise is stimulated by decreasing blood glucose concentrations during periods of rest and exercise, reduced CHO uptake and oxidation by skeletal muscle should reduce the reliance on hepatic production of glucose to maintain euglycemia during rest and exercise (Terjung, 1979). Hence, it was expected that plasma glucagon, cortisol and GH

would all be lower and plasma insulin would be higher during rest and exercise following KS compared with placebo. In support of the hypotheses, insulin was increased and glucagon was decreased in the KS condition compared to placebo, specifically during rest and at 50% VO_2max. On the other hand, cortisol and GH were not different between KS and placebo.

Though the effects of exogenous ketones on glucagon have yet to be reported, the increase in serum insulin observed within this study was in line with previous studies, as the insulinotropic effects of exogenous KB has been reported several times during rest and exercise conditions (Clarke and Cox, 2015; Leckey et al., 2017; O'Malley et al., 2017; Rodger et al., 2017; Evans et al., 2018). Within these studies, an increase in circulating concentrations of insulin and KB coincided with reduced resting concentrations of glucose, lactate and NEFA within 30 min of exogenous ketone ingestion. A decrease in glucose and lactate was not observed during the KS condition within the present study; however, this was most likely a reflection of the participants consuming the KS supplement 30 mins following CHO consumption. In support of this view, when elite cyclists consumed a KE and CHO drink, plasma glucose concentrations during rest and exercise measurement time points were almost identical to when these same athletes consumed a CHO drink alone (Cox et al., 2016). Therefore, the moderate amount of CHO consumed during both the KS and placebo conditions may have attenuated any differences in serum glucose and lactate during the TTE protocol.

Despite observing concentration kinetics in glucose, lactate and NEFA in contradiction to our hypotheses, it must not be overlooked that our hypotheses for glucagon and insulin concentrations during the fatigue measurement time point were also incorrect, with no differences in concentrations being observed between the KS and placebo conditions. It is not completely understood why the balance of these hormone concentrations were changed at

fatigue, though the increased recruitment of highly-glycolytic muscle fibers as participants reached fatigue may have been a factor (Allen et al., 2008). In this case, a heavier reliance on CHO utilization may have been favoured in these muscle fibers, thus stimulating increased utilization of glycogen and/or plasma glucose to fuel mechanical work. NE and Epi were not measured within the present study, however, increased plasma glucose uptake by skeletal muscle would be expected to stimulate the release of NE and Epi from the adrenal glands. These two catecholamines are in turn responsible for reducing the release of insulin, while increasing the secretion of glucagon from the pancreas (Terjung, 1979). Insulin and glucagon concentrations were different between supplementation conditions at 50% VO_2max but consistent at fatigue, therefore, blood NE and Epi concentrations were likely lower following KS compared with placebo at 50% VO_2max but not statistically different between conditions at fatigue.

Additionally, no blood concentration differences in NEFA were seen during the resting and 50% VO_2max measurement time points of the TTE, though NEFA concentrations were significantly increased at fatigue within the KS condition compared to placebo. Previous work with elite cyclists has reported that KE ingestion increased IMTAG utilization during 75% Wmax exercise, therefore, a heavier reliance on IMTAG and plasma glucose utilization by skeletal muscle during the fatigue measurement time point would be expected to increase serum NEFA concentrations (Cox et al., 2016).

Overall, KS plus MCT supplementation was shown to increase performance and reduce rate of perceived exertion during a sub-maximal, fatigue protocol, however, in contradiction with our hypotheses, no performance or RPE differences were seen between KS and placebo conditions during the 10-km TT cycling protocol. Upon further investigation, it was determined that average VO_2 during the TT performance and the 70% VO_2max measurement time point of

the TTE were not statistically different during the placebo condition (Fig. 12F), suggesting that on average, participants were cycling at ~70% VO_2max while completing the TT test. Although no differences were found between placebo conditions, VO_2 was significantly elevated ($p < 0.05$) during the 10-km TT compared to the 70% VO_2max measurement time point of the TTE during KS and MCT supplementation. Terminal oxidation of KB is expected to decrease VO_2 during exercise, therefore, it is speculated that KB utilization was decreased during the TT protocol of the KS and MCT supplementation phase.

Though these findings were in contradiction with our hypotheses, differences in exercise performance during the TTE and TT protocols may have been due to the athletic background of the athletes. Of the 7 athletes recruited, 6 athletes competed in ice hockey where work output during competition is completed in an interval manner, where players complete short, high-intensity on-ice sprints that are followed by periods of extended rest while on the bench. Interestingly, during the TT test, this same interval pattern of work output was observed within these athletes. Instead of pacing themselves, similar to the cross-country athlete used within this study, these ice hockey athletes completed cyclical sets of high-intensity sprints followed by moderate intensity sprints. Based on this high-intensity-interval approach, it is likely that KB utilization was limited when cycling was completed >70% VO_2max during the TT protocol of the KS and MCT supplementation phase (Cox et al., 2016). In support of this theory, during highly glycolytic cycling protocols where CHO oxidation is paramount, VO_2 has been shown to significantly increase following KS ingestion despite identical average power outputs compared to control (Rodger et al., 2017). Although exogenous ketones did not alter performance compared to placebo during these cycling tests, O'Malley et al. (2017) report a ~7% decrease in performance when a 10-km cycling TT was completed following 5 min at 30%, 60% and 90% of

ventilatory threshold. Based upon the need for oxygen to terminally oxidize KB, IMTAG and NEFA, exercise performance may be hindered at cycling workloads > 70% VO_2max (Cox et al., 2016; Evans et al., 2017). As such, the potential for KS plus MCT supplementation to improve performance may be limited to submaximal exercise performances such as ultra-endurance running and cycling events where VO_2 is not a limiting factor for maintaining work output during a competitive race.

Furthermore, in contradiction to our hypothesis, resting VO2 during the TT protocol was increased during KS and MCT supplementation compared to placebo. Since exogenous ketones have previously been shown to reduce glycolytic flux and activity of PDH during rest and exercise, it is plausible that the extra 2-days of KS plus MCT supplementation prior to the TT test caused a significant reduction in activity of PDH. Reduced glycolytic flux would be expected to shift a heavier reliance on terminal oxidation of NEFA and IMTAG to fuel skeletal muscle at rest, thus, the increased VO_2 observed at rest prior to the TT may simply be a reflection of an inability to efficiently produce adequate ATP through aerobic glycolysis, increasing the need for oxygen to terminally oxidize IMTAG and NEFA sequestered from adipose tissue (Cox et al., 2016).

Implications for KS use as an ergogenic aid

Ergogenic aids are attractive for athletes when performance can potentially be improved and/or the rate of perceived exhaustion during performance can be reduced (Cox and Clarke, 2014; Egan and D'Agostino, 2016; Evans et al., 2017). Within this study, KS plus MCTs effectively improved performance on a time to exhaustion cycling test while simultaneously reducing average RPE during the testing protocol. In contrast to many other studies using exogenous ketones, performance gains within this study were also achieved without significant

gastrointestinal side effects, with only one participant reporting gastrointestinal symptoms of a mild stomach ache (Cox and Clarke, 2014; Cox et al., 2016; Leckey et al., 2017; Evans et al., 2018).

Of the two exogenous ketone sources, ketone esters are an attractive ergogenic aid for their ability to substantially increase blood D-BHB concentrations to > 3 mM when ingested alone or to ~ 2 mM when ingested alongside CHOs; however, the mild to severe gastrointestinal symptoms reported following ketone ester ingestion raises concerns for its attractiveness to athletes for use in competition (Cox and Clarke, 2014, Leckey et al., 2017). As such, Leckey et al. (2017) reported that the gut discomfort experienced by world-class cyclists following ketone ester ingestion increased overall perception of effort during a TT performance, with participants reporting a state of mild to severe distraction during cycling. After completing the protocol, when asked who would use the ketone supplement for performance, only 1 out of the 10 total cyclists reported that they might be interested.

Though this response is commonly observed following ketone ester ingestion, similar gastrointestinal symptoms are observed when high doses of KSs are ingested (Evans et al., 2017; Evans et al., 2018). During a graded exercise test, 68% of participants reported gastrointestinal symptoms following two BHB salt doses of 0.38 g/kg body mass, with 7 and 3 participants reporting specific symptoms of nausea and diarrhea during the late stages of a cycling test (Evans et al., 2018). When a BHB salt dose of 0.3 g/kg body mass is ingested, as was used within this study, few to no gastrointestinal symptoms have been reported by participants (O'Malley et al., 2017). Unfortunately, if the overall goal is to elevate and sustain blood D-BHB concentrations to the level of skeletal muscle saturation (~1-2 mM), then a dose higher than 0.3 g/kg body of current KS supplements is needed (Evans et al., 2017; Stubbs et al., 2017). Further

research is required to distinguish the optimal KS supplementation protocol that would increase blood D-BHB concentrations within this range, mitigate adverse gastrointestinal side effects and improve performance. With reference to the results in this thesis, this may include a 6-day supplementation of a KS supplement solely comprised of D-BHB, caprylic acid (C8) and capric acid (C10) and CHO.

Implications for KS use in athletic and competitive performances

Previous TT exercise tests, as well as the TTE test used within this study, suggest that an acute dose of exogenous ketones can elevate blood D-BHB concentrations within ~0.6 to 6 mM following ingestion, subsequently improving exercise performance through alterations in delta and gross efficiency (Clarke and Cox, 2015, Cox et al., 2016). Given these acute changes in performance, it must be acknowledged that exogenous ketones increased exercise performance even when participants were not fully keto-adapted (Volek and Phinney, 2015; Volek et al., 2016, McSwiney et al., 2018). However, similar to the KD, if KB concentrations are elevated but a participant is not fully adapted to using KBs for fuel, then improvements in delta and gross efficiency may be limited following an acute dose of exogenous ketones (Volek et al., 2016).

For many athletes, adhering to a LCKD is very difficult and unattractive due to the severe restrictions on daily CHO intake. Thus, chronic supplementation of exogenous ketones alongside a high CHO diet may provide a unique approach to improve performance without restricting an athletes nutritional intake (Evans et al., 2017). If supplementation is maintained for ≥ 6 days, an athlete may noticeably increase ketolytic enzyme activity while maintaining muscle and liver glycogen stores. Although, high circulating D-BHB and full glycogen stores may be beneficial to performance, inducing this metabolic state may come at the expense of reduced glycolytic flux and activity of PDH in skeletal muscle (Cox et al., 2016, Leckey et al., 2017). As shown

previously, when athletes consumed exogenous ketones prior to completing exercise at >70% VO_2max, reduced performance compared to control has been observed (Leckey et al., 2017; O'Malley et al., 2017; Evans et al., 2018). Therefore, this novel diet may only be advantageous for exercise performances completed at or below 70% VO_2max. Conversely, when athletes participate in highly glycolytic performances such as sprinting or ice hockey, where anaerobic and aerobic glycolysis are almost solely relied upon for ATP production, performance may be limited with KS plus MCT supplementation (Cox et al., 2016, Evans et al., 2017).

Furthermore, research has speculated that exposing the human body to elevated circulating KB concentrations may be most beneficial during ultra-endurance racing events where average exercise intensity during cycling and/or running has been measured to occur near the first ventilatory threshold (Laursen et al, 2005; Volek et al., 2016). This low-moderate workload is a reflection of the total duration to complete an ultra-endurance event, as many competitions include total durations of ~6 to 24 hours. With extended race durations such as these, many ultra-endurance athletes have switched from a high CHO diet to a LCKD in hopes of maximizing KB, FFA and IMTAG oxidation during competition (Volek and Phinney, 2015; McSwiney et al., 2017). Work by Volek et al. (2016), showed that ultra-endurance athletes who adhered to a LCKD for a minimum of 6 months had a 2.3-fold higher peak fat oxidation than athletes adhering to a high CHO diet. Peak fat oxidation also occurred at a higher percentage of VO_2max in LCKD athletes, with peaks at ~70% VO_2max compared to ~55% VO_2max in high CHO athletes. Similarly, Burke et al. (2017) reported that elite race walkers completing 4-weeks of a LCKD increased peak fat oxidation to ~1.9 g/min compared to ~1.5 g/min observed in high CHO athletes. Of note, the latter study saw a decrement in performance with LCKD athletes compared to CHO athletes during a TT test, though Volek et al. (2016) suggests that a LCKD

may need to be maintained for a minimum of 6-months before optimal KB utilization and glucose handling can be achieved. Although the exact time period to achieve optimal KB utilization is still currently unknown, exogenous ketones could play an important role when an athlete beginning a LCKD has a long-term goal of achieving full keto-adaptation (Evans et al., 2017). Regardless of the time frame to achieve adaptation, if the exposure to elevated D-BHB concentrations is the major driver of keto-adaptation, then it is plausible that daily consumption of exogenous ketones could reduce the needed time to achieve full keto-adaptation (D'Agostino et al., 2017). During this period, exogenous ketone supplementation would also be beneficial for athletes consuming LCKD with increased daily totals of CHO (> 20 g) that have been shown to reduce endogenous KB production compared to CHO restrictive (< 20 g) LCKDs (Phinney, 2004; Volek and Phinney, 2015; D'Agostino et al., 2017; Evans et al., 2017).

Moreover, during competition, exogenous ketones may serve a beneficial role during and immediately following exercise (Holdsworth et al., 2017). To maximize KB availability and sustain skeletal muscle saturation and blood D-BHB concentrations (~1-2 mM) during extended ultra-endurance races, ingestion of KSs or KEs may be warranted. In the case of KS specifically, the high salt load accompanied by these supplements may be attractive for athletes who experience significant electrolyte and mineral imbalances during these extended races (Cox et al., 2016; Stubbs et al., 2017). With respect to post-exercise benefits, KEs have been shown to increase the rate of glycogen synthesis following high-intensity exercise, therefore, post-exercise consumption of exogenous ketones may be beneficial during multiple-stage endurance races, where a competitor may be required to complete multiple races within a confined time period (Holdsworth et al., 2017).

Although ultra-endurance athletes may benefit with exogenous ketone supplementation prior to, during and following exercise, Burke et al. report that an acute dose of exogenous ketones may serve little competitive advantage during elite and world-class endurance performances (Burke et al., 2017). Within these shorter competitive races, an athlete is required to complete multiple intervals of highly-glycolytic workloads, such as during hill sprints and the start and finish of a race. Even though KBs can be oxidized at highly glycolytic workloads, reduced PDH activity could hinder an athletes performance when exercise intensity is sustained at workloads > 80% VO_2max (Cox and Clarke, 2014; Cox et al., 2016; Evans et al., 2017,). Consequently, the paucity of exogenous ketone research regarding the potential benefits during 'real-world' exercise tests hinders many athletes from adopting the use of exogenous ketones or a LCKD (Volek and Phinney, 2015; Volek et al., 2016; Burke et al., 2017; Evans et al., 2017). Future research is needed to determine when and how exogenous ketones may be beneficial during 'real-world' competition, and if chronic exogenous ketone supplementation may play a significant role in keto-adapting an athlete.

Limitations

A crucial limitation to this study was that the two supplement drinks used within this study were not isocaloric in nature. As such, performance improvements during the TTE may have been due to excess energy supplied to participants during the KS and MCT supplementation phase compared to placebo. Additionally, the absence of NE and Epi measurements during the TTE test was also a major limitation. While counter-regulatory response speculations have been made, none can be confirmed in the absence of these catecholamine measurements. Additionally, this study did not collect or analyze urine samples, breath acetone concentrations, muscle biopsies and/or use stable isotope tracer molecules that are needed to make accurate evaluations

of relative substrate utilization during rest and exercise (Cox et al., 2016; Evans et al., 2017, Leckey et al., 2017). Based on this, Vmax system measurements were primarily used to understand the potential mechanisms that may have altered skeletal muscle KB utilization between conditions and between cycling protocols. It is also important to note that all participant meals were not standardized during each phase of the study, hence, differences in caloric intake and macronutrient consumption may have altered fingertip blood sampling, venous blood sampling and Vmax system measurements. Although procedures were put in place to limit caloric intake and macronutrient composition differences, the gold standard of dietary control would have included the use of standardized meals during both phases of the study.

Future Directions

Previous research studies have focused heavily on the metabolic effects of exogenous KB ingestion prior to exercise performance, however, the potential for KBs to improve post-exercise recovery has gained recent popularity. During post-exercise recovery, the rate of muscle protein synthesis (MPS) and glycogen synthesis are two major targets for nutritional interventions to temper and regulate (MacDougall et al., 1995; Phillips et al., 1997; Koopman et al., 2005). To limit muscle protein breakdown and promote net MPS, current nutritional interventions prescribe the immediate supplementation of protein and/or amino acids following exercise, while immediate post-exercise consumption of CHOs are recommended for increasing CHO availability and the synthesis of glycogen (Burke et al., 1993; Burke, 1997; Phillips et al., 1997). Although only preliminary data exist, exogenous KBs may play an integral role in post-exercise recovery, as co-ingestion with CHO has been shown to improve glycogen synthesis (Evans et al., 2017; Holdsworth et al., 2017).

In a recent study with military servicemen, Holdsworth et al. (2017), reported that repletion of muscle glycogen following an interval-based glycogen depletion exercise protocol was improved when a dietary ketone ester was consumed in conjunction with a hyperglycaemic clamp. In these well-trained male athletes, when the ketone monoester was combined with a 10 mM glucose clamp, total infused glucose and muscle glycogen content increased by 30% and 50% compared to glucose alone. Moreover, Cox et al. (2016), have shown that glycolysis and the presence of glycolytic intermediate concentrations were significantly reduced when athletes consumed pre-exercise KE drinks versus CHO and FAT drinks. Put together, these two studies propose that exogenous ketones co-ingested alongside CHO may be an effective strategy to attenuate glycolytic flux and direct glucose towards storage as glycogen during post-exercise recovery.

With respect to protein and exogenous KB co-ingestion, limited research exists concerning the direct effects of hyperketonemia on MPS. Despite limited reports, previous exogenous ketone work by Nair et al, has shown that sodium D-BHB infusion decreased leucine oxidation by at least 18% and increased skeletal muscle uptake of leucine by a minimum of 5% when [D-BHB]s of ~2 mM were achieved (Nair et al., 1988). The findings of this study were comparable with a study by Sherwin et al., (1976) where sodium D-BHB infusion decreased both alanine concentrations and urinary nitrogen excretion by 30% while subjects were in a fasted state. Put together, these studies raise the possibility that co-ingestion of exogenous ketone salts alongside protein, amino acid and CHO supplements may be the most optimal post-exercise nutritional strategy during recovery for purposes of increasing the rate of glycogen repletion and attenuating amino acid oxidation (Evans et al., 2017).

In addition to the potential for D-BHB to alter post-exercise recovery, D-BHB may also play a crucial role as a signalling molecule. Independent of terminal oxidation within skeletal muscle mitochondria, it has recently been shown that exogenous D-BHB may also function as a signalling molecule within living organisms, interacting with signalling pathways related to oxidative stress, inflammation, longevity and energy expenditure (Veech et al., 2017).

Firstly, D-BHB may stimulate upregulation of the mitochondrial antioxidant defence system through histone deacetylase (HDAC) inhibition. Class I and II HDACs are enzymes that remove acetyl groups from lysine amino acids on histone molecules, subsequently allowing histones to tightly wrap DNA. Opposing these enzymes, histone acetyltransferases are enzymes responsible for transferring acetyl groups to conserved lysine amino acids on histone proteins. Expression of DNA is thus regulated by the activity of both histone deacetylases and histone acetyltransferases (Gregoretti et al., 2004; New et al., 2012). Specific to the effects of D-BHB, mouse tissues exposed to exogenous D-BHB exhibited increased histone acetyltransferase activation leading to increased transcriptional activation of forkhead box O3A (FOXO3A) and metallothionein 2 (MT2), two specific genes known to combat the oxidative stress response (Shimazu et al., 2013). HDAC inhibition via D-BHB has yet to be confirmed within human skeletal muscle, though D-BHB is speculated to play a similar oxidative defence role as shown in mouse kidneys treated with exogenous D-BHB (Newman and Verdin, 2014).

D-BHB may also play a minor role in the longevity of living organisms through the regulation of forkhead box transcription factor (FOXO) activity. Hyperketonemia, achieved through D-BHB administration or sustained adherence to a KD, has been shown to inhibit insulin-mediated glucose uptake and attenuate insulin/insulin-like growth factor receptor signalling (ISS) (Freeman et al., 1998; Veech et al., 2001; Wheless, 2004). Attenuation of ISS

reduces downstream kinase activity of PI3K and AKT, consequently decreasing phosphorylation of FOXO proteins. In their unphoshporylated form, FOXO proteins remain in the nucleus where they can enhance the gene expression of cellular defence antioxidant enzymes (Brunet et al., 2001; Stitt et al., 2004). Presently, it is not fully understood whether decreased phosphorylation of FOXO proteins is a consequence of decreased ISS or D-BHB inhibition of class I and IIa histone deacetylases, but regardless of the mechanism, further research using D-BHB administration will warrant the potential therapeutic use for improving cellular defence against oxidative stress (Newman and Verdin, 2014).

Likewise, D-BHB may also be potentially therapeutic for cellular stimulation of autophagy. Mammalian target of rapamycin (mTOR1), a major inhibitor of autophagy, is known to be activated through the upstream AKT signalling pathway (Sarbassov et al., 2005). In line with the notion that D-BHB inhibits AKT signalling, conditions of hyperketonemia may stimulate autophagy through inactivation of mTOR1 and subsequent activation of autophagy-inducing proteins (Ravikumar et al., 2004; Kim et al., 2011). It is also hypothesized that D-BHB can induce autophagy through an alternative pathway, via activation of FOXO proteins through the AMPK signalling pathway (Veech et al., 2017). Composed together, it is hypothesized that hyperketonemia established via exogenous D-BHB may provide a unique tool to extend the life span of humans (Evans et al., 2017; Puchalska et al., 2017).

Furthermore, D-BHB may also play a crucial role within chronic inflammation, specifically with a potential role within the neuro-inflammatory response. During the aging process, as well as during periods of lipotoxicity and depression, NLRP3-driven inflammation is proportionally increased (Shao et al., 2015). Within rodent models, exogenous D-BHB administration has been shown to reduce symptoms of neuro-inflammation through regulation of

TNF-alpha and IL-1Beta (Youm et al., 2015). Similarly in human monocytes, BHB attenuated production of IL-1beta and IL-18 following administration (Goldberg et al., 2017). Mechanistically, D-BHB inhibition of the NLRP3-inflammasome has been shown to be independent of starvation-regulated mechanisms and D-BHB oxidation within the TCA cycle, yet the exact mechanism by which D-BHB inhibits inflammasome activation remains unclear at present (Youm et al., 2015).

Lastly, D-BHB may alter energy expenditure within living organisms through interaction with G-protein-coupled receptors. Within rodent models, D-BHB was shown to suppress SNS activity through interaction with receptor GRP41, and when bound to nicotinic acid receptor GPR109A, has shown the potential to inhibit lipolysis in adipocytes and promote secretion of adiponectin (Kimura et al., 2011; Fu et al., 2015). These evolutionarily-conserved signalling responses are still advantageous within human species today, as tempering lipolysis of adipose tissue and suppressing SNS activity would conserve valuable energy stores and limit ketogenesis during periods of full starvation (Veech et al., 2017).

Conclusion

In conclusion, this thesis study reports that KS plus MCT supplementation increases mechanical efficiency, decreases RPE and improves performance during a 70% VO_2max TTE cycling protocol in male varsity athletes. When KS and MCT were supplemented twice-daily for 8 days, performance was not improved on a 10-km cycling time trial test compared to placebo, therefore, the potential for KS plus MCT supplementation to improve performance may be limited to exercise performances ≥ 50% VO_2max and ≤ 70% VO_2max. Additionally, at rest and during cycling exercise at 50% VO_2max, KS and MCT supplementation was shown to blunt the counter-regulatory response to exercise, however, no differences between KS or placebo

supplementation were observed during exercise at 70% VO_2max. Based on the potential role of D-BHB as both an energy substrate and signaling molecule, it is proposed that KS and MCT supplementation may play a meaningful role within competitive performances (Evans et al., 2017; Puchalska et al., 2017, Veech et al., 2017).

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