KETONE MONOESTER SUPPLEMENTATION AND EXERCISE EFFICIENCY

THE EFFECTS OF ACUTE KETONE MONOESTER SUPPLEMENTATION ON EXERCISE EFFICIENCY AND THE INFLUENCE OF DOSE AND INTENSITY

By JACK BONE, B.Sc. Kin

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TITLE: The Effects of Acute Ketone Monoester Supplementation on Exercise Efficiency and the Influence of Dose and Intensity**.** AUTHOR: Jack Bone B.Sc. Kin (Wilfrid Laurier University) SUPERVISOR: Dr. Martin Gibala NUMBER OF PAGES: x, 64

Lay Abstract

Endurance exercise performance is determined by many variables including the efficiency of the individual. This can be measured during cycling by calculating the ratio of oxygen uptake relative to power output. Ketone supplements have been suggested to alter exercise efficiency. We investigated this issue by having trained adults complete an incremental cycling protocol on three occasions. Before exercise the participants ingested either a small or large dose of a ketone supplement or a taste-matched placebo drink. Exercise efficiency was not different between the conditions but ventilation rate and heart rate were higher during the ketone supplemented trials compared to the placebo. The power output that the participants could achieve at maximal exercise was reduced in the high dose ketone condition. Our study does not support the use of ketone supplements as a strategy to enhance endurance exercise performance. Future studies should investigate the mechanisms by which ketones affect exercise responses.

Abstract

Introduction: Acute ketone monoester (KE) supplementation affects exercise responses but there are equivocal data regarding the effects on exercise efficiency. We examined the effect of ketone monoester ingestion on exercise efficiency during cycling and probed further the influence of supplement dose and exercise intensity. This study was registered prior to data collection at ClinicalTrials.org (NCT05665855).

Methods: Twenty-eight trained adults were recruited [16 males, 12 females; peak oxygen uptake ($\dot{V}O_2$peak): 59±11 ml·kg^{-1}·min^{-1}]. Participants completed three experimental trials in a randomized, crossover, and double-blinded manner, each separated by ~1 week. Participants ingested either a 0.3 (KE-LO) or 0.6 g/kg (KE-HI) body mass dose of KE or a flavour-matched placebo (PLAC) ~30 min prior to exercise. The incremental cycling protocol involved a 3-minute warm-up, three 5-minute stages at 75%, 100%, and 125% of individual ventilatory threshold, and a ramp increase to volitional exhaustion. Expired gases and heart rate were measured continually during exercise.

Results: Venous blood [ß-hydroxybutyrate], the major circulating ketone body, was higher in both KE conditions compared to PLAC and also different between conditions (3.0±1.1 and 2.3±0.6 vs 0.2±0.1 mM; all $p<0.05$). There were no differences in submaximal exercise $\dot{V}O_2$, exercise economy, gross efficiency, or delta efficiency between conditions (all $p>0.05$). Submaximal exercise heart rate and ventilation were higher in both KE conditions compared to PLAC (141±11 and 141±12 vs 137±12 beats/min; 63±14 and 62±13 vs 60±13 L/min, respectively; all $p<0.05$). Peak power output at $\dot{V}O_2$peak was lower in KE-HI compared to both KE-LO and PLAC (329±60 vs 339±62 and 341±61 W; both $p<0.05$).

Conclusion: KE supplementation did not alter exercise efficiency during submaximal cycling. KE ingestion increased cardiorespiratory stress during submaximal exercise and the higher dose reduced peak aerobic power output. Future studies should investigate the mechanisms by which KE ingestion alters exercise responses.

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List of Abbreviations

ABBREVIATION	TERM
KB	Ketone body
KE	Ketone ester
KS	Ketone salt
ßHB	ß-hydroxybutyrate
ACAC	Acetoacetate
BD	1,3-butanediol
OXCT	Succinyl-CoA-3-oxaloacid CoA transferase
ACAT	Acetyl-CoA acetyltransferase
MCT	Monocarboxylate transporter
MPC	Mitochondrial pyruvate carrier
TCA CYCLE	Tricarboxylic acid cycle
ATP	Adenosine triphosphate
EE	Energy expenditure
PDH	Pyruvate dehydrogenase
PFK	Phosphofructokinase
P/O RATIO	Ratio of ATP produces per mol of O_2 used
GE	Gross efficiency
DE	Delta efficiency
VT_1	First ventilatory threshold
$\dot{V}O_2$PEAK	Peak oxygen uptake
PPO	Peak power output

Declaration of Academic Achievement

Format and Organization of Thesis:

This thesis is prepared in the standard format as outlined in the School of Graduate Studies' Guide for the Preparation of Master's Theses. The first chapter is a literature review and the second chapter is a draft of a manuscript for submission to a journal.

List of Contributors to Thesis:

J. Bone[1], S. Baumgarten[1], D. G. McCarthy[1], W. Bostad[1], D. L. Richards[2], M. J. Gibala[1]

Departments of Kinesiology[1] and Medicine[2], McMaster University, Hamilton, Ontario L8S 4K1, Canada.

Breakdown of Contributions:

J. Bone and M.J. Gibala formulated the research question and study design with input from D. G. McCarthy. J. Bone completed data collection with help from S. Baumgarten, W. Bostad, and D. G. McCarthy, input from M.J. Gibala, and medical oversight from D. Richards. J. Bone performed data analysis with input from M.J. Gibala. J. Bone and M.J. Gibala interpreted the study results with input from D. G. McCarthy. J. Bone formulated and wrote initial drafts of the literature review and manuscript (Chapter 1 and 2) with input and assistance from M.J. Gibala. All authors will have the opportunity to review and approve the final version of the manuscript prior to submission to a journal

Chapter 1:

1.1 Introduction

Humans have long endeavored to enhance athletic performance through various means including adherence to specific dietary practices and the ingestion of supplements (Burke & Hawley, 2018). One strategy that has re-emerged in popularity in recent years is the ketogenic diet. By restricting carbohydrate (CHO) intake, the approach is meant to increase the natural production of endogenous ketones bodies (KB) to use as a fuel source and alter substrate oxidation. KB are lipid-derived hydrocarbon molecules produced naturally in the liver and so named for containing a ketone functional group (Robinson & Williamson, 1980). The most physiologically relevant ketone bodies are β-hydroxybutyrate (βHB), acetoacetate (AcAc), and acetone. Apparently healthy individuals who ingest a typical mixed diet have a resting blood KB concentration of ~0.2 mM (Robinson &Williamson, 1980). Nutritional ketosis refers to a state characterized by a blood KB level of >0.5 mM (Poff et al, 2020). This can be induced through adherence to a ketogenic diet which involves ingesting ≤50 g of CHO per day for ≥3 weeks (Burke, 2021). However, generally the low CHO intake associated with a ketogenic diet is generally not deemed beneficial in sport or events that necessitate a high rate of carbohydrate oxidation to optimize performance (Burke, 2021).

Exogenous KB supplements have emerged as a novel method to rapidly elevate blood KB in humans without restricting CHO intake. Ingestion of exogenous ketone supplements can elicit nutritional ketosis and induce significant increases in blood ketone levels, depending on dosage and supplement type (Margolis & O’Fallon, 2019). It has been suggested that KB supplementation offers the potential to improve exercise performance by altering substrate oxidation, although the precise mechanisms remain equivocal (Sato et al, 1995; Veech et al, 2004; Petrick et al, 2020; Dearlove et al, 2021). The effect of KB supplementation on exercise performance is another topic of considerable interest in the field, but the studies to date are equivocal (Cox et al, 2016; Leckey et al, 2017; Margolis & O'Fallon, 2019; Poffé et al, 2021b; McCarthy et al, 2023b).

One factor that influences exercise performance is efficiency, which is broadly defined as the ratio of energy output to energy input (MacDougall et al. 2022). Owing to its relative simplicity, the measurement of oxygen uptake ($\dot{V}O_2$) is the most common method of measuring the energy cost of exercise and thus efficiency, particularly at submaximal intensities below the second lactate threshold (MacDougall et al. 2022). A related variable is gross efficiency, which is used usually as a measure of whole-body efficiency (Moseley & Jeukendrup, 2001; MacDougall et al, 2022). Another is delta efficiency, which has historically been thought to reflect muscular efficiency (Gaesser & Brooks, 1975), although this point is contentious (MacDougall et al. 2022). It has been reported that ketone monoester supplementation may affect exercise efficiency by influencing $\dot{V}O_2$ during submaximal exercise (Dearlove et al, 2021, Evans et al, 2022a). However, there remains a dearth of research in this field, and the mechanisms by which this change in exercise efficiency may occur are not well understood. As a means of exploring this topic, this chapter provides an overview of (a) KB metabolism in healthy humans, (b) physiological responses to KB supplementation at rest and exercise, (c) the various measures of exercise efficiency, and (d) explore how these factors may interact to influence exercise responses.

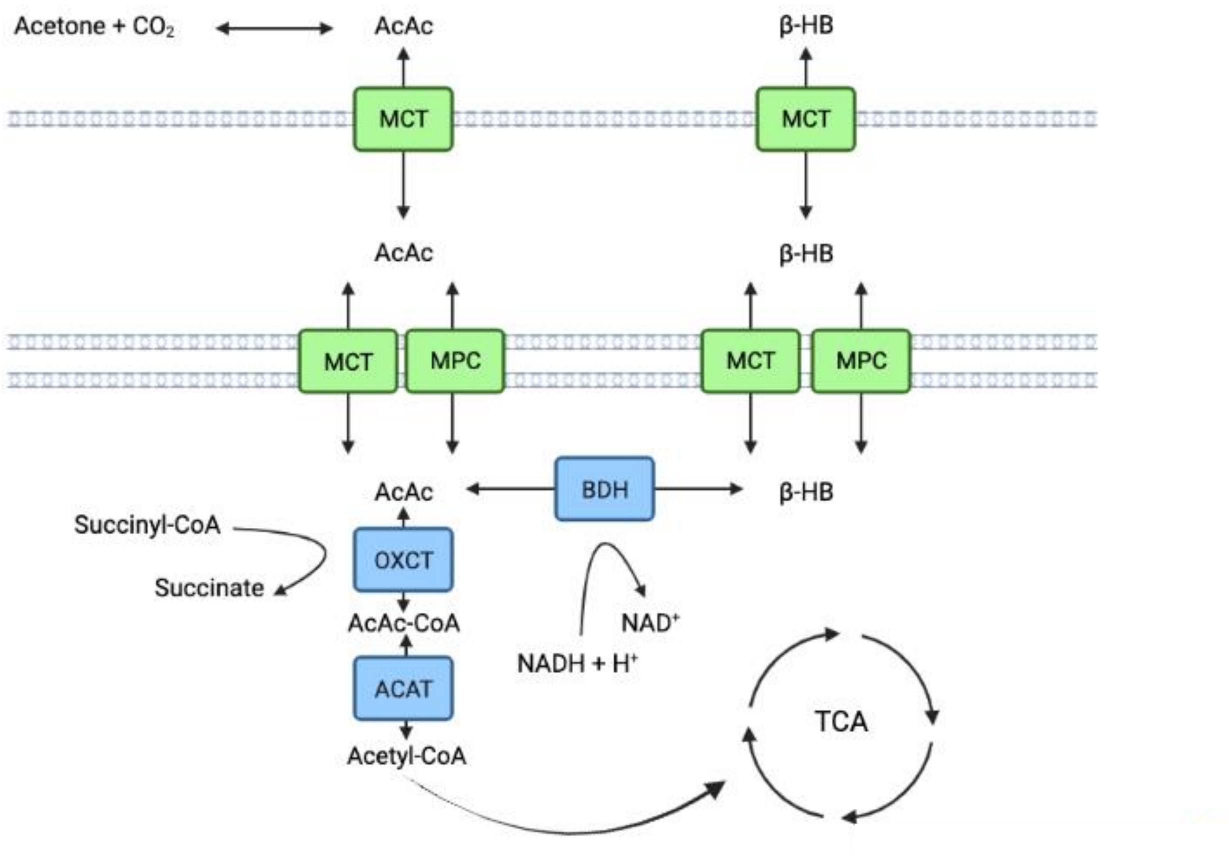
1.2) Overview of Ketone Body Metabolism

1.2.1) Ketone Body Production and Inter-Organ Exchange

The concentration of plasma KB is reflected by the interaction between KB production in hepatic tissues (ketogenesis) and KB catabolism in extra-hepatic tissues such as skeletal and cardiac muscle (ketolysis) (Robinson & Williamson, 1980; Evans et al, 2017). Ketogenesis plays a role in survival as an adaptive response to severe energy restriction, as the brain is unable to utilise free-fatty acids (FFA) as substrates (Evans et al, 2017). The principal KBs are AcAc, acetone, and βHB (Robinson & Williamson, 1980); βHB is not technically a KB but is metabolically linked to the others and is therefore commonly referred to as one (Pinckaers et al, 2017) including for the purposes of this review.

FFAs are the most common substrates for ketogenesis, with the breakdown of amino acids such as leucine, isoleucine, and lysine also providing small amounts (Evans et al, 2017; Thomas et al, 1982). Elevations in FFA released from adipose tissue occur with increased rates of lipolysis, as well as other characteristics of severe CHO restriction such as low plasma glucose and insulin levels (Evans et al, 2017). FFAs are taken up by hepatocytes, the site of ketogenesis. Here, multiple enzyme-driven reactions take place to ultimately produce acetoacetate, which can have three fates: directly enter the bloodstream via monocarboxylate-transporter (MCT); decarboxylate into acetone and carbon dioxide (CO_2); or be reduced by βHB dehydrogenase into βHB, which also exits the hepatocytes via MCT into the bloodstream (Robinson & Williamson, 1980; Evans et al, 2017; Soto-Mota et al, 2020). An overview of these processes is shown in Figure 1.

Figure 1: Overview of ketone body metabolism in extra-hepatic tissues. AcAc: acetoacetate; βHB: β-hydroxybutyrate; CO_2: carbon dioxide; MCT: monocarboxylate transporter; MPC: mitochondrial pyruvate carrier; BDH; βHB hydrogenase; CoA: coenzyme-A; OXCT: succicyl-CoA-3-oxaloacid CoA transferase; AcAc-Coa; acetoacetate-CoA; ACAT; acetyl-CoA-acetyltransferase. Modified from Evans et al (2017), created with BioRender.com.



After reaching target tissues, KBs are transported into mitochondria via MCT or mitochondrial pyruvate carriers (MPCs), where they undergo the reverse reaction catalysed by βHB dehydrogenase, being converted back into AcAc while producing NADH (Shaw et al, 2020; Evans et al, 2017; Soto-Mota et al, 2020). The rate-limiting enzyme of ketolysis, succinyl-CoA-3-oxaloacid CoA transferase (OXCT), then converts

AcAc and succinyl-CoA into succinate and AcAc-CoA, which acetyl-CoA acetyltransferase (ACAT) then cleaves into two molecules of acetyl-CoA. These are then incorporated into the tricarboxylic acid (TCA) cycle via citrate synthase for production of ATP (Petrick et al, 2020; Evans et al, 2017; Soto-Mota et al, 2020). These extra-hepatic tissues contain KB enzymes and are therefore able to utilise them as energy substrates (Petrick et al, 2020). In summary, KB are produced in the liver, circulated through the bloodstream before being taken up and utilised by tissues such as skeletal and cardiac muscles.

1.2.2) Ketone Body Oxidation

The activity of OXCT is highest in the heart and kidneys, but skeletal muscle accounts for the highest fraction of KB oxidation at rest owing to its far larger proportion of total body mass (Robinson & Williamson, 1980; Balasse & Féry, 1989; Evans et al, 2017). KBs can be used as additional energy substrates, like fats and CHO (Mikkelsen et al, 2015; Petrick et al, 2020). However, their relative contribution to total energy expenditure (EE) is much lower under typical resting conditions, with KB oxidation accounting for ~2 - 10% of total EE, while FFAs accounting for most of the energy provision (Balasse & Féry, 1989; Evans et al, 2017). This percentages rises substantially after fasting and starvation, demonstrating a curvilinear relationship with respect to concentration, with KB providing ~10% EE after an overnight fast, ~20-50% after 72 h, but declining to ~15% after 24 d of starvation (Owen & Reichard, 1971). This indicates that KB uptake into skeletal muscle is governed by saturation kinetics, as shown by

Balasse & Féry (1989). Petrick et al (2020) also demonstrated *in vitro* that while KBs can drive respiration in skeletal muscle, their contribution to metabolism is low.

As the metabolism of KBs provide only acetyl-CoA, whereas pyruvate can yield acetyl-CoA and oxaloacetate, a TCA cycle intermediate, it has been suggested that KBs be ingested alongside glucose-derived products to aid in their oxidation (Kadir et al, 2023). While βHB oxidation has been found to increase in perfused rat hearts with higher glycogen content (Kadir et al, 2023), many complex metabolic interactions could affect the metabolism of KBs. As KBs produce NADH and acetyl-CoA at different stages of metabolism, the production of NADH and acetyl-CoA from other sources, such as pyruvate, could inhibit these reactions (Petrick et al, 2023). While in the presence of saturating pyruvate, the ability of KBs to stimulate mitochondrial oxidation is impaired, producing levels of ATP up to three times lower than pyruvate (Petrick et al, 2020). Competition exists between KBs and other substrates used in oxidation, shown through the addition of KBs and pyruvate to permeabilized muscle fibres, where the presence of pyruvate impaired the ability of βHB to stimulate cellular respiration (Petrick et al, 2020). However, AcAc was not affected in the same way, suggesting that skeletal muscles may have a greater capacity to oxidize AcAc compared to βHB (Petrick et al, 2020).

KBs have been proposed as a more efficient source of energy when compared to CHO and fats (Veech, 2014). This is owing in part to βHB having a greater theoretical energy-yield than pyruvate (~13.0 vs 10.0 ATP per carbon) (Veech, 2014; Burgess et al, 2008; Shaw et al. 2019), although this comparison is likely less appropriate than one to glucose (12.7 ATP per carbon) due to the majority of pyruvate in skeletal muscles being broken from glucose. βHB also possess more Gibbs free energy to perform work than glucose and pyruvate (Sato et al, 1995; Veech et al, 2004). One commonly cited study used as an example for the energetic benefits of KBs showed a significant (25%) improvement in the hydraulic efficiency after KBs of a working rat heart after KBs were added it (Sato et al, 1995). ATP per produced oxygen (O_2) molecule (P/O ratio) may be an even more appropriate method of comparison. As O_2 is consumed whenever ATP is produced during oxidative phosphorylation, a higher P/O ratio would indicate greater efficiency. Theoretically, 1 mol of both βHB and acetoacetate would yield the same ratio of 2.5 ATP/O_2 (Karwi & Lopaschuk, 2022), which is greater than that of fatty acids at a P/O raito of 2.33. However, the oxidation of glucose and muscle glycogen results in an ATP/O_2 ratio of ~2.58 ATP/O_2 (Karwi & Lopaschuk, 2022). Therefore, glucose may be just as if not more efficient at producing energy when compared to KBs. This, along with research probing the extent that KB oxidation contributes to energy expenditure, has implications on the effects of KBs on exercise responses and performance and will be explored later in this review.

1.2.3) Nutritional Ketosis

Nutritional ketosis refers to a state characterized by a blood KB level of >0.5 mM (Poff et al, 2020). When the rate of KB production in the liver exceeds breakdown and rate of disappearance, blood [KB] levels increase over the course of several metabolic stages, with the initial rise being attributed to higher rates of ketogenesis in hepatic tissues due to FFA spillover (Féry & Balasse, 1986). The subsequent increases in blood [KB] can be attributed to decreases in ketolysis and the utilization of KBs in peripheral tissues such as skeletal muscle, a consequence of KB feedback inhibition caused by the saturation kinetics of muscles with KB uptake (Reichard et al, 1971; Balasse & Féry, 1989). The ketogenic diet is unique among dietary interventions for eliciting nutritional ketosis as it is the only form of CHO restriction that allows the consumption of food. This allows for a prolonged state of nutritional ketosis not otherwise possible with different methods of CHO restriction such as long-term fasting and starvation. Participants adhering to a ketogenic diet have demonstrated blood KB levels that usually do not exceed ~4 mM, although there have been reports of up to ~8 mM (Volek et al, 2015; Phinney et al, 1983; Pinckaers et al, 2017; McCarthy et al, 2021). In summary, various methods of CHO restriction can induce ketosis to varying degrees and precise "dose-response" effect between blood KB the effect on KB metabolism is unclear.

1.24) Effect of Nutritional Ketosis on Metabolism

As a potential source of ATP, it is possible that the oxidation of CHO and fats may be reduced when blood KB is elevated. CHO and fat oxidation generally show an inverse relationship, i.e. when fat oxidation is high, CHO oxidation is low, and vice versa (van Loon et al, 2001; Burke et al, 2017). This can be determined via a variety of non-invasive, reliable methods such as indirect calorimetry. Interpretations from indirect calorimetry are based off the assumption that all carbon dioxide produced comes from the oxidation of fats and CHO. This may be violated in a state of nutritional ketosis due to the production of CO_2 during the oxidation of KB and when acetoacetate is decarboxylated into acetone (Reichard et al, 1979; Soto-Mota et al, 2020). Substrate oxidation can also be determined using stable isotope tracers and mass spectrometry, although this is a more invasive method. Currently, there are limited data on how increasing blood KB might change fat and CHO oxidation in humans during exercise. Nutritional ketosis has been shown to have several effects on the metabolism of CHO and fats demonstrating a concomitant decrease in blood concentration of free fatty acids and glucose, either when KB are introduced intravenously or through supplementation (Balasse & Ooms, 1968; Ari et al, 2019, Mikkelsen et al, 2015). KBs may also have a direct effect on substrate oxidation regardless of changes to circulating concentrations of fatty acids and glucose (Beylot et al, 1986), however the methods by which the results were achieved may not be applicable to athletes in natural settings.

1.3) Exogenous Ketone Body Supplementation

When KB are ingested as a supplement, the metabolism differs from endogenous KBs in that the KBs must go through the digestive tract before being absorbed in the bloodstream. There are also several different kinds of KB supplements with different chemical compositions and effects on KB concentration. Thus, it is important to review the availability of these different KB supplements, their digestion, and how they affect metabolism, both at rest and during exercise.

1.3.1) Types of Ketone Body Supplements

Several types of exogenous KB supplements are available including ketone monoesters, ketone salts, and ketone (specifically acetoacetate) diesters (Stubbs et al, 2017; Leckey et al, 2017; Stubbs et al, 2018). 1,3-butanediol (BD) is a precursor to βHB that has been used alone as a supplement in several exercise studies as well (Shaw et al, 2019). These KBs and KB components differ in several ways, primarily in terms of their chemical makeup. Ketone monoesters are composed of D-B-hydroxybutyrate in addition to BD, while D/L-B-hydroxybutyrate and $Na^+/K^+/Ca^+$ are what makes up a ketone salt (Clarke et al, 2012a; Stubbs et al, 2017). Finally, an acetoacetate diester is made up of two acetoacetate molecules alongside D/L 1,3-butanediol (Leckey et al, 2017; Stubbs et al, 2017). Each of these supplements are commercially available. Ketone monoesters have consistently been shown to increase blood βHB to the greatest extent, up to threefold that of identical doses of ketone salts (Stubbs et al, 2017), and routinely raising blood KB above ~2 mM (Margolis & O'Fallon, 2019). Ketone salts normally see the smallest increase, rarely increasing to above 1 mM, whereas acetoacetate diesters and BD see concentrations of ~1.0 – 1.5 mM (Margolis & O'Fallon, 2019).

1.3.2) Effect of Ketone Body Supplementation on Metabolism

KB supplements are primarily ingested in a liquid form and pass through the mouth without any kind of digestion via enzymes present in the oral cavity. Notably, KB and butanediol have an inherently bitter taste, which may activate certain receptors in the mouth and/or throat. This bitter flavour may stimulate a gag reflex and lead to vomiting, alongside general gastrointestinal distress, a potential side effect after KB ingestion (Stubbs et al, 2019). The addition of CHO and caffeine with or after KB ingestion may serve to mask the bitter flavour. These practices can increase the activity of regions of the brain associated with pleasure and reward by activating oral taste receptors (Wickham & Spriet, 2018; Chambers et al, 2009). The KB supplements then enter the stomach, before moving into the small intestine. The process of gastric emptying of KBs into the small intestine is not well understood. While there have been no direct measures of the specific effects of KBs on gastric emptying to date, general principles suggest that increasing the osmolality of a beverage slows gastric emptying rate (Vist & Maughan, 1995; Horner et al, 2015). Thus, as a beverage with a KB supplement would have a higher osmolality compared to one with no KBs, then a KB supplement could in theory slow gastric emptying rate, although this would not be due to the KBs specifically. More research should be done on this topic to determine exact effects.

Once in the small intestine, KB supplements are metabolized in different ways depending on their composition. Ketone esters are cleaved by esterase enzymes in the gut into their components (such as butanediol and βHB for a ketone monoester), which are then taken up by the liver (Shivva et al, 2016; Soto-Mota et al, 2020; Shaw et al, 2020). Here they undergo metabolism just as endogenous KBs would, although butanediol is metabolized into βHB via alcohol dehydrogenase first (Soto-Mota et al, 2020; Shaw et al, 2020). Ketone salts are dissociated into their components, including βHB, which is transported and metabolized in the same fashion as βHB from the monoesters (Shivva et al, 2016; Shaw et al, 2020).

Current research on the pharmacokinetics of KBs after ingestion suggest that all types of supplements elevate blood βHB within 30 min after ingestion, with levels remaining elevated for up to several hours afterwards (Stubbs et al, 2017; Leckey et al, 2017; Shaw et al, 2019). Relative to dose, ketone monoesters have consistently been shown to result in the greatest increase in blood βHB after consumption, with participants regularly achieving nutritional ketosis at levels >2 mM (McCarthy et al, 2021; Stubbs et al, 2017; Poffé et al, 2021a; Cox et al, 2016; Margolis & O'Fallon, 2019). Ketone salts, acetoacetate diesters, and butanediol also consistently have participants reach nutritional ketosis, but to a lesser magnitude, often peaking at ≤1.0 mM blood βHB (Margolis & O'Fallon, 2019). Despite βHB being the prime measurement for ketosis, blood acetoacetate levels also increase after ingestion of KB supplements, meaning researchers are often ignoring a significant portion of circulating blood KBs during measurement (Cox et al, 2016; Stubbs et al, 2017). The reasons for this are primarily practical, due to the degradation of AcAc in stored blood samples and the relative ease of measurement of βHB through point of care analysis (Price et al, 1977; McNeil et al, 2014). Blood AcAc levels are still important for overall nutritional ketosis and the pharmacokinetics of unique KB supplements, and as such should be considered when determining how certain supplements affect ketone levels.

1.3.3) Physiological Responses to Nutritional Ketosis During Exercise

There are many potential ways in which KBs may influence exercise responses after nutritional ketosis has been induced (Figure 2). Regarding possible ergogenic effects, three key mechanisms have been postulated. The first is as a potential additional energy substrate, theoretically providing additional ATP for exercise (Mikkelsen et al, 2015; Petrick et al, 2020). Second, KBs may generate ATP in a more 'cost-effective' manner compared to ATP produced through the oxidation of fat and CHO (Sato et al, 1995; Veech et al, 2004; Dearlove et al, 2021). Finally, KBs could reduce reliance on CHO utilization during exercise, resulting in a glycogen 'sparing effect' (Evans et al, 2017; Shaw et al, 2020). However, multiple studies have also reported evidence to suggest that KBs may be ergolytic in nature, resulting from mild to severe gastrointestinal

(GI) distress, increased cardiorespiratory stress, and impairing tissue lipolysis and CHO utilization (Cox et al, 2016; Leckey et al, 2017; Evans et al, 2018; Poffé et al, 2020; McCarthy et al, 2021; Poffé et al, 2021a; Poffé et al, 2021b; McCarthy et al, 2023b).

KBs may have beneficial effects related to recovery through actions as signalling molecules (Newman & Verdin, 2017; Evans et al, 2022a). Anti-inflammatory and anti-catabolic qualities have been observed (Koutnik et al, 2019; Stubbs et al, 2020), and two recent studies found significant increases in serum erythropoietin with KE supplementation post-exercise (Evans et al, 2022b; Poffé et al, 2023). While exploring these findings is outside the scope of this review and the following section, their impact on the future of the field merits mention. The following section will review the potential exercise responses to nutritional ketosis after acute KB supplementation.

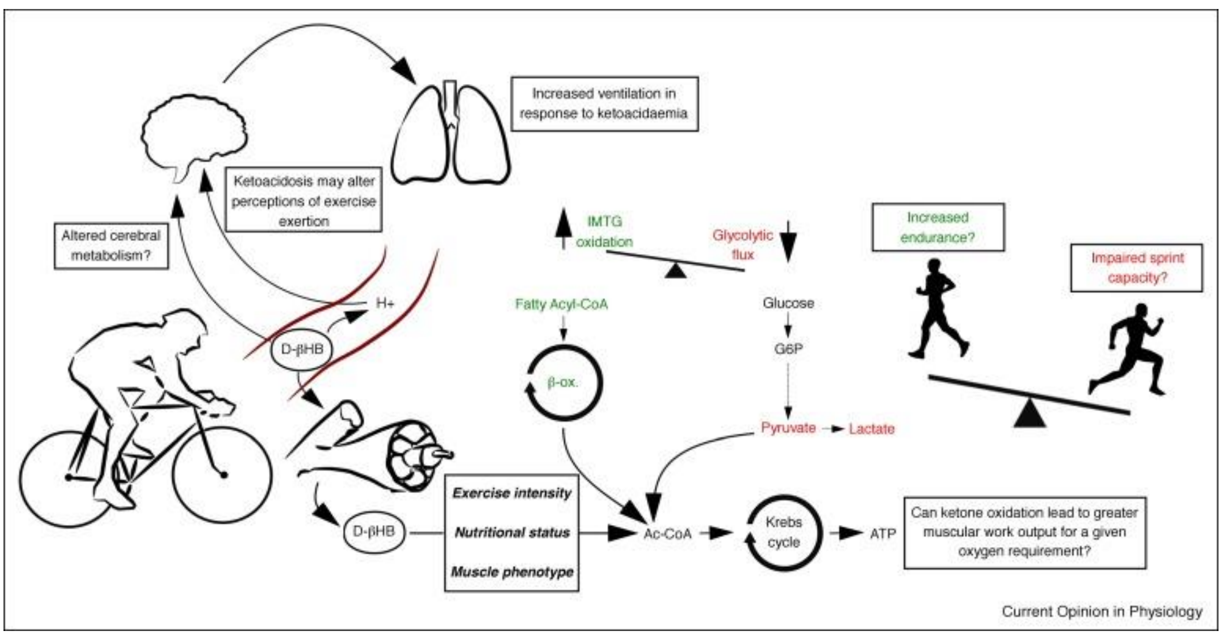


Figure 2: Purported and hypothesised effects of exogenous ketone consumption during exercise. Exercising skeletal muscle oxidises ketone bodies, which may be thermodynamically advantageous compared to glucose and fat oxidation by increasing the Gibbs free energy for ATP synthesis. The intensity of exercise performed, the nutritional

status of the muscle (fed versus fasted) and the muscle phenotype may influence ketone oxidation rates. Exercise substrate metabolism is altered during exogenous ketosis, with increased fat oxidation and decreased glycolytic flux. Glycogen sparing and potential improvements in energy transduction through ketone oxidation may modestly increase long duration exercise performance. However, high-intensity exercise that relies on rapid glycolytic flux and anaerobic glycolysis may be negatively affected. Exogenous ketosis may beneficially alter cerebral metabolism, potentially delaying central fatigue, although this remains speculative. Exogenous ketosis results in a mild ketoacidaemia, which is compensated for by an increased ventilatory drive. Leg discomfort, anxiety of breathlessness and anxiety of leg discomfort are increased during ketoacidosis. Ac-CoA: acetyl CoA; ATP: adenosine triphosphate; β-ox.: β-oxidation; D-βHB: D-β-hydroxybutyrate; H^+: hydrogen ion; IMTG: intramuscular triacylglycerol; G6P: Glucose-6-Phosphate. Figure reproduced from Dearlove et al (2019b) with use and attribution in accordance with Creative Commons license.

1.3.4) Potential Ergogenic Mechanisms Related to Nutritional Ketosis

The potential KBs to act as an additional substrate for energy provision was a focus of relatively early literature that considered the utilization and metabolism of KBs during exercise (Balasse & Ooms, 1968; Féry & Balasse, 1986; Balasse & Féry, 1989). Briefly, these studies found a significant increase in the oxidation of KBs, under the presumption that KB rate of disappearance was equivalent to oxidation (Féry & Balasse, 1986; Balasse & Féry, 1989). However, KBs did not contribute significantly to energy provision, at ~2-10% (Balasse & Féry, 1989). Recent studies using indirect methods of estimating the oxidation rates of KBs (Cox et al, 2016) as well as labelled tracers (Dearlove et al, 2021; Howard et al, 2023) have reported that KBs may contribute as little as ~3% to overall energy provision during exercise to as high as ~18%, with the lower end being more in agreement with the values reported in earlier studies (Balasse & Ooms, 1968; Féry & Balasse, 1986; Balasse & Féry, 1989). It is generally accepted in the literature that ketone bodies contribute ≤5% to total energy expenditure (Evans et al, 2017; Evans et al, 2022) A recent study of permeabilized muscle fibres and mitochondria observed that KBs contribute minimally to cellular respiration, especially in the presence of other substrates such as pyruvate (Petrick et al, 2020). Due to their contribution to energy provision during exercise, however minor, there is research to suggest that this may result in the sparing of glycogen, which potential ergogenic results (Evans et al, 2017; Shaw et al, 2020). KE supplements have been observed to increase the contribution of fatty acids to energy provision as well, potentially sparing glycogen even further (Cox et al, 2016). However, research on the exact effects of KBs on this aspect remain equivocal and need to be studied in greater depth.

Despite the likely minor contribution to energy provision during exercise, KBs may be a more efficient source of ATP compared to CHO or fat, as seen earlier in this review. Most studies examining the effects of KBs on energetic efficiency examine myocardial physiology, presenting an issue that any improvements seen may not translate to working skeletal muscle (Karwi & Lopaschuk, 2022). If KB supplementation were to improve exercise efficiency through the proposed mechanisms, it would manifest as a lower $\dot{V}O_2$ while exercising at identical power outputs, or a higher power output at identical levels of $\dot{V}O_2$. While neither have been seen to date, a recent study did find an improvement in a measure of exercise efficiency influenced by $\dot{V}O_2$ (Dearlove et al, 2021), which will be discussed further in a subsequent section on the effects of KB supplementation on exercise efficiency later in this review.

1.3.5) Potential Ergolytic Mechanisms Related to Nutritional Ketosis

Not all exercise responses to KB supplementation are purported to be ergogenic in nature. Some of the most common and oft-cited issues with KB supplementation are the GI symptoms associated with their ingestion, including bloating, flatulence, cramping, belching, vomiting, and diarrhea (Leckey et al, 2017; Evans et al, 2018). Several studies using KS have seen high incidences of GI symptoms during exercise with acute doses of 36 to 48 g, although this may be a dose response as smaller doses have not been associated with such issues (Evans et al, 2018; Shaw et al, 2019; Stubbs et al, 2019; Jo et al, 2022). A study using ketone diesters reported significant GI symptoms (Leckey et al, 2017), while ketone monoesters have also been reported to result in the same issues when participants ingested doses of 32 to 60 g (Evans et al, 2018; McCarthy et al, 2021). However, studies using similar doses of KE have seen no such issues with GI symptoms (Stubbs et al, 2019; Poffé et al, 2020; Poffé et al, 2021a), and these effects have been shown to lessen or not manifest if KB supplements are split into multiple, smaller doses as opposed to being ingested as one large bolus (Evans et al, 2019).

Both βHB and AcAc are weak organic acids, and therefore induce a mild blood acidosis (~0.05 – 0.10 lower pH) when nutritional ketosis is reached. The associated increase in H^+ ions is considered ergolytic as it limits the buffering capacity needed during high intensity exercise (MacDougall et al, 2022). These responses also come with increases in CO_2, subsequently inducing an increase in minute ventilation as evidenced by various studies (Poffé et al, 2021a; McCarthy et al, 2021; McCarthy et al, 2023a). Co-administration of bicarbonate has been explored as a strategy to mitigate the decrease in blood pH, with some success (Poffé et al, 2021c; McCarthy et al, 2021). Notably, Poffé and colleagues (2021) found that the co-ingestion of KE and bicarbonate increased average power output by ≤5% in a 15 min TT after a 3-hour pre-load when compared control as well as KE and bicarbonate by themselves.

As previously mentioned, it has been speculated that KBs may elicit a glycogen sparing effect. However, it may be that KBs do not so much as "spare" but rather "impair" the utilization of CHO by inhibiting glycolysis, as seen in several mammal studies using canine and rodent tissue (Maizels et al, 1977; Laughlin et al, 1994). This would be done through the inhibition of key metabolic enzymes involved in CHO oxidation, such as phosphofructokinase (PFK) and pyruvate dehydrogenase (PDH), achieved through increases in NADH/NAD^+, acetyl-CoA and/or citrate through the metabolism of acetoacetate (Randle et al, 1964; Maizels et al, 1977; Laughlin et al, 1994). A recent tracer study found impaired lipolysis and glycolysis during exercise in both healthy human adults and those with McArdle disease after exogenous KE supplementation (Løkken et al, 2022). Data from studies on low-CHO high-fat diets support the notion that impairment of CHO utilization is ergolytic, especially at high intensities (Burke, 2021). While there have been several KB supplementation studies where high-intensity exercise performance was impaired, it is unknown if the mechanism of this change was due to KB on CHO utilization. Circulating FFA levels may be reduced upon the achievement of acute nutritional ketosis, suggesting that KBs may inhibit adipose tissue lipolysis (Cox et al, 2016; Poffé et al, 2020).

1.4) Effects of Ketone Bodies on Exercise Performance

In a now seminal study, Cox and colleagues (2016) reported a 2% improvement in cycling time trial performance in trained cyclists after a 0.573 g/kg dose of ketone monoester that increased blood βHB concentrations to ~2-2.5 mM. Since this publication, other studies that have explored the effects of KB supplementation on exercise performance have reported divergent results. Many of these studies differ greatly in their methodological designs, with variations in supplement type and dosage, exercise protocol, and participant training state and ability. A summary of this work is presented in the following section.

Since the original study reporting an improvement in cycling TT performance (Cox et al, 2016), there have been several experiments which also report the performance enhancing potential of KBs. Kackley and colleagues (2020) found that the ingestion of a mixed supplement containing 7.2 g of KS improved time to exhaustion in an incremental cycling test in both keto-adapted and naïve recreationally active adults. However, plain water was used as a placebo, and the mixed drink contained ~100 g of caffeine as well as L-taurine and L-leucine, so the improvements in exercise performance cannot be solely attributed to the KBs. A study in professional rugby players also found that increasing circulating blood KB concentration to ~2 mM through co-ingesting a 0.59 g/kg KE dose with CHO improved the average time to completion of a high intensity performance test compared to just ingesting CHO (Peacock et al, 2022).

A recent publication found ergogenic responses to KEs during a 10-day period of daily supplementation which included 6 days of a simulated endurance race (Dearlove et al, 2022). Trained endurance athletes were separated into three groups, each consuming a different diet: either high in CHO, a high-CHO diet with four daily weight-adjusted KE drinks, or a ketogenic diet. Pre- and post-intervention testing consisted of 90 min of cycling at 70% $\dot{V}O_2$max followed by an incremental time to exhaustion test. This same protocol was done in the morning of each of the simulated race days, where the KE drink group performed significantly (6-8%) better on days 1, 4, and 6 on the time to exhaustion test compared to pre-intervention performance, whereas the CHO group only improved on day 1 (5%) (Dearlove et al, 2022). However, the relatively small sample size (n = 7 per group) and the fact that the race tests were performed in a fed state as opposed to the fasted state of the pre-intervention test to which they were compared are methodological issues that limit the veracity of the conclusion regarding an ergogenic effect.

As summarized in recent reviews, most research regarding performance shows either neutral or ergolytic effects (Margolis & O'Fallon, 2019; Brooks et al, 2022). Some of these studies used ketone salts (KS) as their method of inducing nutritional ketosis, with O'Malley and colleagues (2017) finding that ingesting 0.3 g/kg of KS 50 min prior to a simulated 150 kJ TT increased blood βHB to ~0.8 mM and impaired performance in said TT, decreasing average power output by 16 watts and increasing time to completion. Another found no difference in 4 min power output after highly trained cyclists ingested two 30 ml (11.7g) doses of KS 30 min prior to and 45 min into a 90 min pre-load at 80% VT (Rodger et al, 2017). Most research in the field use KE in their supplementation protocols (Evans et al, 2018; Evans et al, 2019; McCarthy et al, 2021; Poffé et al, 2021a; Poffé et al, 2021b). While one study did use a ketone diester supplement, nutritional ketosis was not achieved after ingesting two 0.25 g/kg bolus doses 50 and 30 min before a

31.2 TT, with blood βHB only reaching ~0.4 mM. Mean power output was still decreased by ~3.7%, likely attributable to increased gastrointestinal distress (Leckey et al, 2017).

Two recent studies showed reduced exercise performance after the ingestion of KE (Poffé et al, 2021b; McCarthy et al, 2023b). In the first, participants ingested 25 g of KE 30 and 55 min into a 60 min preload of intermittent intensity cycling immediately before a 30 min TT followed by a maximal sprint effort at 175% lactate threshold. The performance of said sprint effort was not affected, but TT performance decreased by ~1.5% (Poffé et al, 2021b). Our own group saw a ~7% decrease in 20 min cycling TT performance after ingesting a 0.35 g/kg dose of KE ~30 min prior (McCarthy et al, 2023b). Other works saw similar results, such as no improvements to 10km running TT performance (Evans et al, 2019) or in a ~20 min incremental cycling test in trained cyclists (Dearlove et al, 2019a). Ultimately, there is little evidence to support KBs as having use as ergogenic aids. However, the equivocality of the research on the subject merits further exploration, given the heterogeneity of study designs. It is possible that under certain circumstances, KBs may prove to be ergogenic, however this context remains elusive.

1.5) Exercise Efficiency and the Influence of Ketone Supplementation

Exercise efficiency is the ratio of work done to energy expended during exercise (Gaesser & Brooks, 1978). In the discipline of exercise physiology, this is commonly expressed as $\dot{V}O_2$ relative to work rate, e.g. cycling workload or running speed (Gaesser & Brooks, 1978; MacDougall et al, 2022), and is known colloquially as exercise economy, which can be thought of as one of many measures of exercise efficiency. Other such measures include delta efficiency and gross efficiency (Gaesser & Brooks, 1978; Moseley & Jeukendrup, 2001), all of which are variables that affect exercise performance (Bassett & Howley, 2000). Similarly, there are many variables that influence these measures themselves, such as cycling cadence and power output (Coyle et al, 1992; MacDougall et al, 2020). Exercise efficiency is influenced by both oxidative and non-oxidative energy provision, and is tied to factors such as motor unit recruitment as well as muscle fiber type (MacDougall et al, 2022).

1.5.1) Physiology of exercise efficiency

During exercise, motor units in our skeletal muscle system are recruited to produce force, and this recruitment pattern follows a 'size principle' – that is, upon the commencement of exercise, smaller motor units comprised of slow-twitch (type I) fibres are recruited first, followed by larger units made up of fast-twitch (type II) fibres as the intensity of the exercise increases (Bawa et al, 1984; Adam & De Luca, 2003). Slow-twitch fibres are thought to have both a higher peak efficiency and better economy of force maintenance compared to fast-twitch fibres (Barclay, 1996; Barstow et al, 1996). However, this notion is disputed by other research, and considerations need to be made regarding the maximal shortening velocity (V_{max}) of the different fibre types as well. Fast-twitch fibres have a much higher (faster) peak shortening velocity compared to slow-twitch fibres. The power-velocity and power-efficiency relationship is similar for both types, with peak power output being achieved around 20-30% of V_{max}, and peak efficiency occurring at a velocity just below that (Barclay, 1996). As such, despite potentially having a lesser peak efficiency compared to slow-twitch fibres, the efficiency of fast-twitch fibres is substantially higher at higher contraction rates (MacDougall et al, 2022). This is if especial importance with respect to cycling efficiency, as cycling is one of few exercise modalities in which power output and rate of contraction can be changed through changing work rate and pedaling rate, or cadence.

The impact of cadence on cycling efficiency lies in the power-velocity relationship of muscle fibres and how motor recruitment patterns change as work rate changes. As a muscle's highest efficiency occurs at a shortening velocity just below that which elicits peak power output, efficiency should decrease if shortening velocity either exceeds or is far below the optimum for power output (Barclay, 2015). As a result, cycling at low intensities with a low cadence would be most efficient for slow-twitch fibres, and cycling at a high cadence with higher power outputs would be most efficient for fast-twitch fibres. As is discussed below, this is accurately reflected by GE, supporting the notion that it may serve as a better means of determining muscular efficiency compared to the classically used delta efficiency (MacDougall et al, 2022).

1.5.2) Measures of exercise efficiency

Exercise efficiency is a variable that has been identified to be an important factor in determining cycling performance (Bassett & Howley, 2000; MacDougall et al, 2022). Within the scope of exercise efficiency lies many different measures, each used to determine different types of exercise efficiency, from whole-body to that focused on the musculoskeletal system (Gaesser & Brooks, 1975; MacDougall et al, 2022). Each measure has strengths and weaknesses, with the validity and reliability of some of these measures being contentious. The simplest measure of exercise efficiency used in the research of exercise responses is $\dot{V}O_2$, which is used as a proxy for energy cost of exercise (MacDougall et al, 2022). The importance of $\dot{V}O_2$ in reference to exercise efficiency is such that it is a common variable used in most other exercise efficiency calculations, such as gross efficiency and delta efficiency. Therefore, any physiological factor that affects $\dot{V}O_2$, of which there are many, will in turn affect these other measures. One such measure of exercise efficiency is exercise economy, simply calculated as the ratio of $\dot{V}O_2$ and work output. Economy is heavily correlated with exercise performance, demonstrating a strong correlation (r=0.82) with 10-kilometre performance time in runners (Conley & Krahenbuhl, 1980; Bassett & Howley, 2000). While its simplicity means greater ease of use and measurement, it lacks the specificity of both gross efficiency and delta efficiency.

Delta efficiency is meant to reflect the mechanical efficiency of the musculoskeletal system during exercise. It is typically calculated as the inverse of the slope from the individual linear regression (y = mx + b) of the relationship between energy expended per minute versus work accomplished per minute (Coyle et al, 1992). It is then calculated from the reciprocal of the slope of this relationship (1/m) and expressed as a percentage. Delta efficiency has historically been purported to be the most effective method of determining the mechanical efficiency of the musculoskeletal system (Poole et al, 1992), suggested to accurately describe the relationship between energy expenditure and work rate (Gaesser & Brooks, 1975).

A review by MacDougall and colleagues (2022) has questioned whether delta efficiency is in fact the best method of representing muscular efficiency. For example, the linearity of the regression line on which it is based is often violated by the slow component of $\dot{V}O_2$, which tends to occur at intensities as low as 50% $\dot{V}O_2max$ (Poole et al, 1992; MacDougall et al, 2022). Additionally, there is an assumption when calculating delta efficiency that work efficiency is independent of work rate (Ettema & Loras, 2009). The authors highlighted a study conducted by Poole and colleagues (1992), which demonstrated that measured delta efficiency of leg $\dot{V}O_2$ during exercise did not agree with leg gross efficiency. Specifically, delta efficiency continually increased and was unaffected by work rate, whereas gross efficiency changed and started increasing at a lower rate as work rate increased (Poole et al, 1992; MacDougall et al, 2022). As was discussed above, muscular efficiency likely changes with work rate, so delta efficiency being unable to reflect such a change calls its validity as a measure of muscular efficiency into question.

MacDougall and colleagues (2022) instead suggest that gross efficiency may serve as a more valid measure of muscular efficiency compared to delta efficiency despite it historically having been used to determine whole-body mechanical efficiency as opposed to muscular. The reasoning for this suggestion is due to the observations made by Poole and colleagues (1992) as well as those made by Coast & Welch (1985), which demonstrated low gross efficiency when cycling at low intensities with a high cadence, but higher gross efficiency when cycling at low intensities and low cadences. This is representative of the musculoskeletal system due to the difference in shortening velocities between the fast and slow twitch muscle fibres making up working muscles which was discussed earlier in this review. However, this is a recent assertion and requires further exploration and consideration.

1.5.3) Influence of Ketone Supplementation on Exercise Efficiency

The effects of KB supplementation on exercise responses remains an equivocal subject, and the interaction between KBs and exercise efficiency is even more so. It may enhance energy efficiency, attributable to the purported increase in energetic efficiency that KBs provide as energy substrates (Sato et al, 1995; Veech et al, 2004). This was reportedly reflected in the 2021 study conducted by Dearlove and colleagues which reported a 7% increase in delta efficiency. However, a limitation of this study was the small sample size (n = 6) which increases the risk of a type II error. The "significant" result reported was also based on an exploratory post-hoc analysis, with no main effect of condition found for delta efficiency (p=0.098) (Dearlove et al, 2021). While this is the only study which directly explores the effects of KE supplementation on exercise efficiency, this influence can also be probed through observing exercise $\dot{V}O_2$ at identical workloads, or workloads at identical relative rates of oxygen uptake. Most studies exploring such effects have seen no changes (Evans et al, 2019; Prins et al, 2020; Dearlove et al, 2021; McCarthy et al, 2021). However, an increase in submaximal oxygen consumption was seen during a 3 h bout of intermittent cycling after ingesting of a 75 g dose of KE, (Poffé et al, 2021a), while our own lab found that a 0.6 g/kg body mass dose of KE decreased peak power output at $\dot{V}O_2$peak compared to control (McCarthy et al, 2023a), both results being indicative of impaired exercise efficiency. As such, there are conflicting results as to the effects of KE supplementation on exercise efficiency.

While the energetics of KBs seem to suggest that they might improve exercise efficiency, once ingested and nutritional ketosis has been achieved, that may not be the case. Energetically, even if KBs were to improve exercise efficiency by reducing $\dot{V}O_2$ as efficient energy substrates, they likely contribute little to energy provision during exercise (Balasse & Ooms, 1968; Féry & Balasse, 1986; Balasse & Féry, 1989; Dearlove et al, 2021). There are also several methods by which KBs could increase $\dot{V}O_2$, thereby resulting in a reduction in exercise efficiency. Although KBs do possess a higher P/O ratio compared to fatty acids, theirs is still lower than that of glucose (Karwi & Lopaschuk, 2022). Therefore, should KBs indeed replace CHO as an energy substrate during exercise, this would result in an increase in $\dot{V}O_2$, albeit a small one given their seemingly minor contribution to energy provision. The increase in the provision of fatty acids to energy expenditure seen with KE supplementation during exercise (Cox et al, 2016) would also reduce exercise efficiency through increased $\dot{V}O_2$ by way of the higher P/O ratio of fatty acids compared to glucose. The mild acidosis and increase in H^+ ions associated with nutritional ketosis induces an increase in minute ventilation as evidenced by multiple studies (McCarthy et al, 2021; Poffé et al, 2021a). As the respiratory muscles are working at a higher rate because of this, $\dot{V}O_2$ would increase slightly. This in addition to any increases in $\dot{V}O_2$ seen from the replacement of glucose as an energy substrate could in theory result in an increase in $\dot{V}O_2$ significant enough to impair exercise efficiency such that exercise performance is reduced.

1.6) Purpose

In summary, the interaction between KB supplementation and exercise efficiency warrants further examination. The primary goal of this thesis was to clarify the effect of KE ingestion on exercise efficiency in humans as primarily reflected by whole-body oxygen uptake during cycling. It was hypothesized that KE ingestion would alter exercise efficiency during submaximal exercise. This effect will be probed further by examining responses to two different doses of KE and at three submaximal workloads that correspond to relatively low, moderate, and high-intensity exercise.

Chapter 2:

Introduction

There has been substantial recent interest in the potential for exogenous ketone body (KB) supplements to alter exercise responses (Margolis & O'Fallon, 2019; Pinckaers et al, 2017, Shaw et al, 2020; Evans & Egan, 2018, Evans et al, 2017; McCarthy et al, 2021). This is related to the fact that acute KB ingestion can induce nutritional ketosis without otherwise altering diet or severely restricting carbohydrate intake (Cox et al, 2016). It has been theorized that acute KB supplementation may improve exercise performance through various mechanisms but findings to date are equivocal. KB ingestion has been reported to improve (Cox et al, 2016; Poffé et al, 2019), impair (O'Malley et al, 2017; Poffé et al, 2021b; McCarthy et al, 2023b), or otherwise have no effect on performance (Evans et al, 2017; Prins et al, 2020; McCarthy et al, 2021). The disparate findings are likely owing to many factors including the dose of KB ingested, training state of the participants, and specific exercise conditions.

Endurance exercise performance is largely determined by the highest rate of oxygen uptake that can be sustained during a given activity, i.e. the "performance $\dot{V}O_2$" (Joyner & Coyle, 2008). Another factor that affects performance is exercise efficiency, which is broadly defined as the ratio of energy output to energy input (MacDougall et al. 2022). The measurement of $\dot{V}O_2$ relative to work rate (e.g. speed or power) — typically called exercise economy — is one of the most common methods of measuring efficiency, particularly at intensities below the second lactate threshold (MacDougall et al. 2022). Other related indices include gross efficiency, which is a measure of whole-body efficiency (MacDougall et al, 2022), and delta efficiency, which has been used to reflect muscular efficiency (Gaesser & Brooks, 1975), although this point is contentious (MacDougall et al. 2022). Ketone monoester (KE) supplementation has been reported to improve delta efficiency, at least at relatively low as compared to high doses, i.e. ~0.25 vs ~0.75 g/kg body mass (Dearlove et al., 2021). It was suggested that this response may be due to exogenous ketone body oxidation resulting in lower oxygen consumption at identical workloads compared to placebo (Dearlove et al., 2021).

There is also evidence that exogenous KE supplementation may elicit responses that could impair exercise efficiency. Ingesting a relatively large dose of KE (0.6 g/kg body mass) prior to exercise has been found to increase ventilation (Dearlove et al, 2019a; McCarthy et al, 2021; McCarthy et al, 2023a) and heart rate (McCarthy et al, 2021; McCarthy et al, 2023a), which are indicative of higher cardiorespiratory stress. This may in part be due to acid-base disturbances, with nutritional ketosis causing a decrease in blood [pH], subsequently increasing ventilation and the work of breathing (Poffé et al, 2020; McCarthy et al, 2021; Poffé et al, 2021a; McCarthy et al, 2023a). This increase in cardiorespiratory stress could reduce exercise efficiency, although the limited number of studies that have measured submaximal exercise $\dot{V}O_2$ have generally reported no difference compared to placebo (Poffé et al, 2021c; Dearlove et al, 2019a; Dearlove et al. 2021). Peak power output at peak oxygen uptake ($\dot{V}O_{2peak}$) has also been shown to be reduced after KE ingestion (McCarthy et al. 2023a), which is indicative of reduced efficiency at high work rates.

The primary purpose of the present study was to clarify the effect of ketone monoester (KE) ingestion on exercise efficiency in humans as primarily reflected by whole-body $\dot{V}O_2$ during submaximal cycling. We hypothesized that KE ingestion would change gross efficiency as determined by power output expressed relative to $\dot{V}O_2$, i.e. cycling economy. We sought to further probe this effect by examining responses to two different doses of KE and at three submaximal workloads that correspond to relatively low, moderate, and high-intensity exercise.

Methods

Participants: A calculation performed using G*Power (version 3.1.9.7) for a one-way repeated measure analysis of variance (ANOVA) to determine an effect of condition revealed that a total sample size of 28 participants provided 80% power to detect a change in $\dot{V}O_2$ at an alpha level of 0.05 with a medium effect size *f* of 0.25. A total of 34 participants were recruited for the study, some of whom were lost to follow up, which resulted in a final sample size of 28 (16 males, 12 females; Figure 3). The participant characteristics are summarized in Table 1. The study inclusion criteria were being an adult aged 18-60 years, deemed safe to engage in physical activity based on the completion the Canadian Society for Exercise Physiology Get Active Questionnaire (https://csep.ca/2021/01/20/pre-screening-for-physical-activity/), habitually consuming ≥50 g/d of carbohydrate (i.e., not following a ketogenic diet), engaged in endurance-type exercise ≥3h/wk, and having an estimated $\dot{V}O_2$peak of ≥90% for age and sex (American College of Sports Medicine, 2000) as determined by an online calculator (https://www.worldfitnesslevel.org).

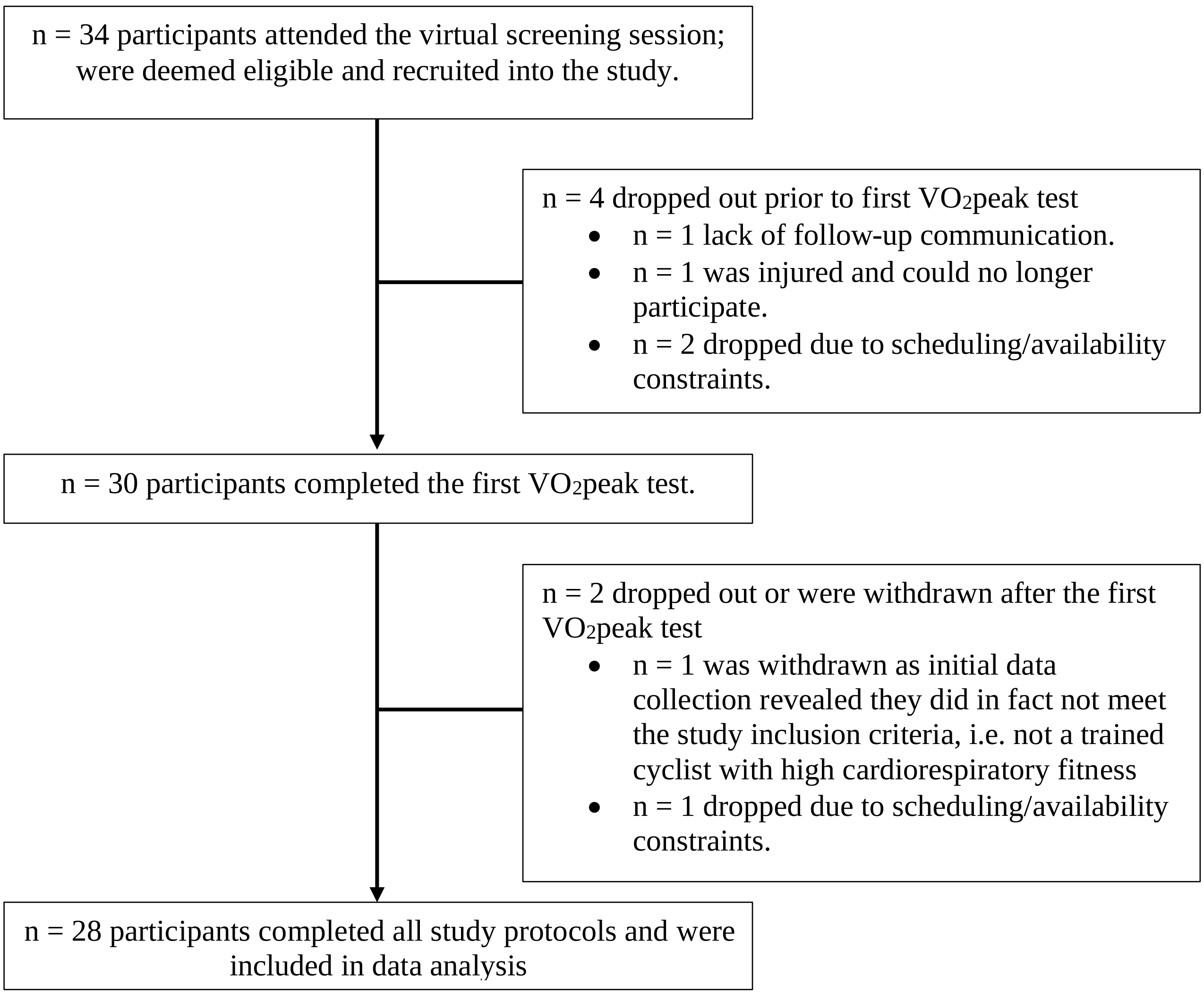
Table 1: Baseline participant characteristics

	Males (n=16)	Females (n=12)	Combined (n=28)
Age (years)	33 ± 11	30 ± 10	27 ± 7
Mass (kg)	75 ± 10	70 ± 11	62 ± 7
$\dot{V}O_2$peak (L/min)	4.7 ± 0.7	4.1 ± 1.0	3.2 ± 0.3
$\dot{V}O_2$peak (ml·kg^{-1}·min^{-1})	64 ± 11	59 ± 11	52 ± 7
Peak power output (W)	388 ± 43	349 ± 60	296 ± 30
HRpeak (beats·min^{-1})	181 ± 12	181 ± 11	181 ± 9

Participants were recruited from McMaster University and the surrounding community with information about the study being shared using printed posters, word of mouth, and social media posts. Interested individuals were informed of the study requirements and provided with an electronic copy of the informed consent form. Participants who satisfied the inclusion criteria and wished to participate were asked to provide written consent and were subsequently recruited into the study. The study protocol was approved by the Hamilton Integrated Research Ethics Board and registered prior to data collection at Clinicaltrials.org (identifier: NCT05665855).

Study overview: A randomized, double-blind, placebo-controlled, crossover design was employed to compare responses between three experimental conditions that differed in the drink ingested prior to exercise. The drinks contained either 0.3 g/kg or 0.6 g/kg body mass dose of a commercial ketone supplement (ΔG Ketone Performance; TDeltaS, Thame, Oxfordshire, United Kingdom) or a custom-made ketone-free, taste-matched placebo (McCarthy et al, 2023b). Participants initially made two visits to the lab

Figure 3: Study CONSORT diagram.



to determine their $\dot{V}O_2$peak, and this was followed by a familiarization visit to introduce the exercise protocol to be used in the main experimental trials. At the end of the familiarization visit, participants were randomly given a small sample of one of the supplement or placebo drink in a blinded manner to become familiar with the bitter taste. The order of the experimental trials was randomized, with a die being rolled for each participant, with each number on the die corresponded to one of the six potential treatment orders. The orders were randomly assigned to the participants through systematic rotation, such that 5-6 participants were in each order group. The three main trials were separated by seven days for all participants. For female participants, trials were scheduled in such a way in an attempt to ensure all three experimental trials occurred during either the luteal or follicular phase of the menstrual cycle. All tests were performed at the same time of day for a given participant. Participants were asked to maintain their habitual physical activity and diet throughout the course of the experiment.

During the first laboratory visit, participants arrived had their height and mass measured before completing an exercise test on a cycle ergometer to determine their $\dot{V}O_2$peak (Lode Excalibur Sport V 2.0, Groningen, The Netherlands). Following a 3 min warm-up at a fixed workload of 50 W, a ramp protocol was applied with a linear workload increase of 1 W every 2 s (30 W/min). Pedaling cadence was chosen by the participant and was required to be ≥60 rpm. A recovery phase was performed at 50 W. Gas exchange and ventilatory variables were continuously measured using a metabolic cart (Quark CPET metabolic cart, COSMED, Italy), and heart rate (HR) was monitored continuously (Polar A3, Finland). Data was averaged over 10-s intervals and $\dot{V}O_2$peak was defined as the highest 30-s average over three consecutive intervals. Data-based cut-offs for age-stratified secondary exhaustion criteria based on peak respiratory exchange ratio and age-predicted maximal heart rate (Wagner et al, 2020) were used to verify that the test involved maximal effort. VT_1 was determined using the protocol described by Gaskill et al., (2001). In brief, VT_1 was visually evaluated via the ventilatory equivalent

(VEQ), the excess carbon dioxide ($ExCO_2$), and the V-slope methods. These values were combined and averaged for determination of VT_1 (Gaskill et al, 2001).

Experimental trials: An overview of the trial design is shown in Figure 4. On the day before the experimental trials, participants were asked to refrain from strenuous exercise and avoid consuming alcohol. Caffeine intake was not restricted but participants were instructed to be consistent in the timing and amount ingested. Participants were asked to maintain a similar diet for the day before and the day of the experimental trials to maintain consistent nutritional intake.

Participants arrived in lab ~35 min prior to exercise and ingested the predetermined drink based on their randomization order. Participants then had their body mass measured. Approximately 10 min prior to exercise, a forearm blood sample was obtained from an antecubital vein via venipuncture. The sample was immediately analyzed by a researcher who did not otherwise interact with the participant to ensure that the other investigators remained blinded to the treatment order.

Participants then completed an incremental exercise test on a cycle ergometer (Lode Excalibur Sport V 2.0, Groningen, The Netherlands). Following a 3 min warm-up at 50 W, they completed three 5-min stages at 75%, 100%, and 125% of VT_1. A pedaling cadence of >60 rpm was maintained for the duration of these three stages and did not deviate from their chosen cadence. The three stages were immediately followed by a ramp protocol to volitional exhaustion with a linear workload increase of 1 W every 2 s (30 W/min) with the goal of reattaining $\dot{V}O_2$peak. Heart rate (HR) was monitored continuously during exercise (Polar A3, Finland), and a metabolic cart with an on-line gas collection system (Quark CPET metabolic cart, COSMED, Italy) was used for the continuous determination of expired gases and ventilation. Rate of perceived exertion (RPE) was assessed with the 20-point Borg scale (Borg, 1982) at the end of each stage. Participants cooled down for as long as needed before filling out a custom blinding and gastrointestinal symptom questionnaire.

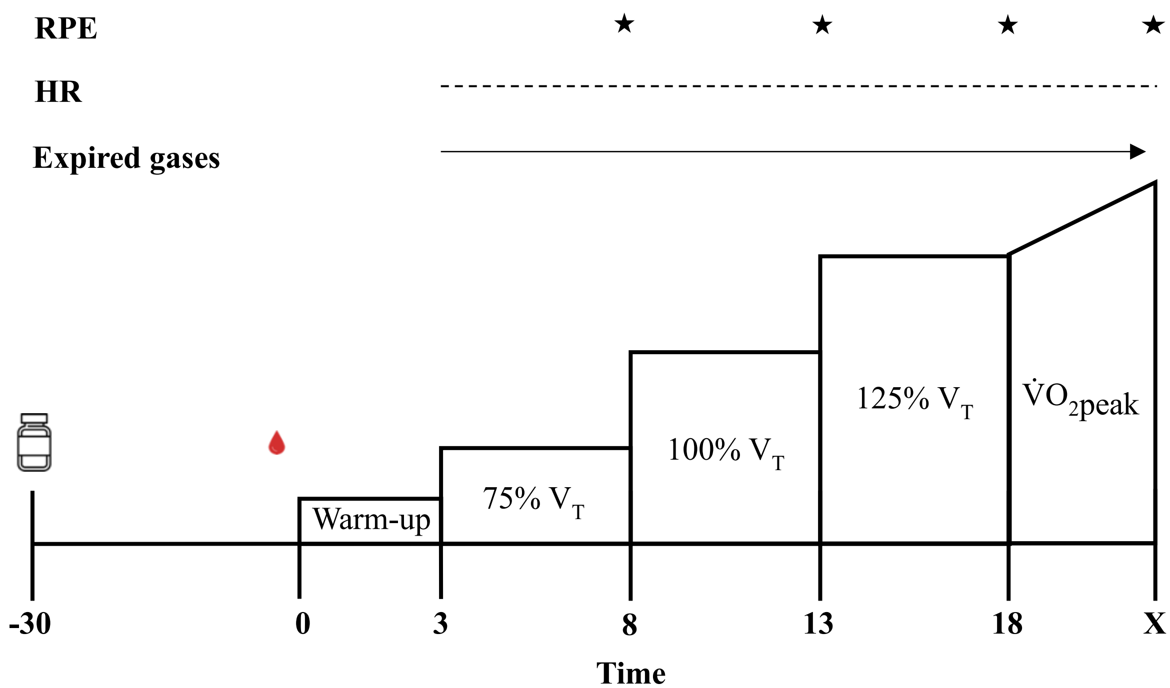


Figure 4: Overview of the experiment protocol. Participants ingested either 0.3 or 0.6 g/kg body mass of the ketone monoester supplement, or a flavor-matched placebo 30 min prior to exercise. A single venous blood sample was obtained just prior to exercise. Exercise involved cycling for 3 min warm-up at 50 W, followed by three 5 min stages at 75%, 100%, and 125% of individual ventilatory threshold, and ended with a ramp protocol to volitional exhaustion. HR, heart rate; RPE, rating of perceived exertion; blood droplet, venous blood draw; numbers, time in min.

Mean submaximal $\dot{V}O_2$, $\dot{V}CO_2$, and ventilation was determined over the 15-min period of submaximal exercise. HRpeak was determined as the highest value achieved during each of the three submaximal exercise stages, and mean HR from the average value across the three stages. $\dot{V}O_2$peak, HRpeak, peak ventilation, and peak power output (PPO) were determined from the final ramp stage, with HRpeak being defined as the highest 2-s average, and PPO as the workload at the time of test cessation. $\dot{V}O_2$peak was determined using the same criteria as in baseline testing.

Blood analyses: A ~1 ml sample was collected into a heparinized syringe and immediately analysed for [glucose], [pH], [lactate] pO_2, pCO_2, and $pHCO_3$ (EPOC, Siemens Healthcare, Ontario, Canada) during each experimental trial prior to exercise. The sample was also applied to a point of care analyzer to measure [βHB] (β-ketone test strips, Freestyle Precision Neo; Abbott Laboratories, IL, USA) in whole blood directly.

Calculations: Gross efficiency was calculated as the mean of all data collected in the final minute of each submaximal stage until the respiratory exchange ratio reaches 1.00 (Moseley & Jeukendrup, 2001). It was calculated as work rate (watts converted to kcal/min) divided by the energy expended (kcal/min) over the exercise bout (Coyle et al, 1992):

$$GE = Work_{(W)} / EE\text{-}GE_{(J/s)}$$

With energy expenditure (EE-GE) being calculated using the following equation as suggested by Brouwer et al (1957):

$$EE\text{-}GE_{(J/s)} = (\{[\dot{V}O_2 \text{ (L/min)} \times 3.869] + [VCO_2 \text{ (L/min)} \times 1.195] \times (4.186 / 60) \times 1000$$

Economy was determined at the same time points as GE, calculated using the following equation:

$$\dot{V}O_2 \text{ (ml)} / \text{Work (W)}$$

Delta efficiency was determined using the same procedure as Dearlove et al (2021), by calculating the individual linear regression (y = mx + b) of the relationship (three data points at 75%, 100%, and 125% VT_1) between energy expended per minute versus work accomplished per minute (Coyle et al, 1992). Delta efficiency was calculated from the reciprocal of the slope of this relationship (1/m) and expressed as a percentage. Energy expenditure for delta efficiency was calculated according to the formulas suggested by Jeukendrup & Wallis (2005):

$$\text{energy expenditure (kcal min}^{-1}\text{) at 25\% and 50\% Wmax} =$$

$$(0.575\ VCO_2) + (4.435\ \dot{V}O_2)$$

$$\text{energy expenditure (kcal min}^{-1}\text{) at 75\% Wmax} =$$

$$(0.550\ VCO_2) + (4.471\ \dot{V}O_2)$$

Respiratory exchange ratio (RER) was calculated as $\dot{V}CO_2/\dot{V}O_2$.

Statistical analyses: Normality was assessed with a Shapiro Wilk's test, and if not normal, tested for lognormality. If data were lognormal, variables were log transformed before testing, and if not, non-parametric tests were performed. A one-way repeated measures analysis of variance (ANOVA) was used to analyze the effect of condition on total $\dot{V}O_2$, exercise economy gross efficiency, delta efficiency, ventilation, heart rate, Wpeak, RPE, blood variables, and gastrointestinal severity. A two-way ANOVA was used to analyze the influence of submaximal exercise intensity on $\dot{V}O_2$, ventilation, and heart rate. Significant main effects and interactions were further analyzed with a Tukey's post hoc test. Participants missing one data point (i.e. from one condition) were included in the analysis, but the data were analyzed with a mixed model ANOVA. If missing two data points, participants data were excluded from the analysis. Statistics were performed with Prism 8 (Graphpad, San Diego, CA, USA) with significance accepted as $p<0.05$. Data are presented as mean ± standard deviation for n=28 except where noted. Non-normal data will be presented as median (interquartile range), change data as mean [95% confidence intervals], and effect sizes as Cohen's d_z. Blood data are based on n=26 as samples could not be obtained from two participants on two trials each.

<u>RESULTS</u>

Blood Data

Blood (ß-hydroxybutyrate) was different between treatments (main effect, $p<0.0001$) such that KE-HI > KE-LO > PLAC (3.0±1.1 vs 2.3±0.6 vs 0.2±0.1 mM; $p=0.0011$ and $p<0.0001$, respectively; Figure 5). All other blood data are reported in Table 2. In brief, blood glucose was 1.0 (0.7-1.3) and 0.7 (0.4-1.0) mM lower in KE-HI ($p<0.0001$, d_z = 1.26) and KE-LO ($p=0.001$, d_z = 0.75) vs PLAC, respectively, and blood pH was 0.021 (0.010-0.032) lower in KE-HI vs PLAC ($p=0.009$, d_z = 0.70). Blood lactate was not different between treatments ($p=0.83$). Blood CO_2 was lower in KE-HI ($p<0.0001$, d_z = 1.77) and KE-LO ($p<0.0001$, d_z =1.10) by 3.6 (1.9-5.3) and 3.2 (0.9-5.5) mmHg respectively. When compared to PLAC, bicarbonate was 3.2 (2.6-3.8) mmHg lower in KE-HI ($p=0.001$, d_z = 0.79) and 2.3 (1.5-3.1) mmHg lower in KE-LO ($p=0.03$, d_z = 0.52). Blood O_2 was not different between treatments ($p=0.13$).

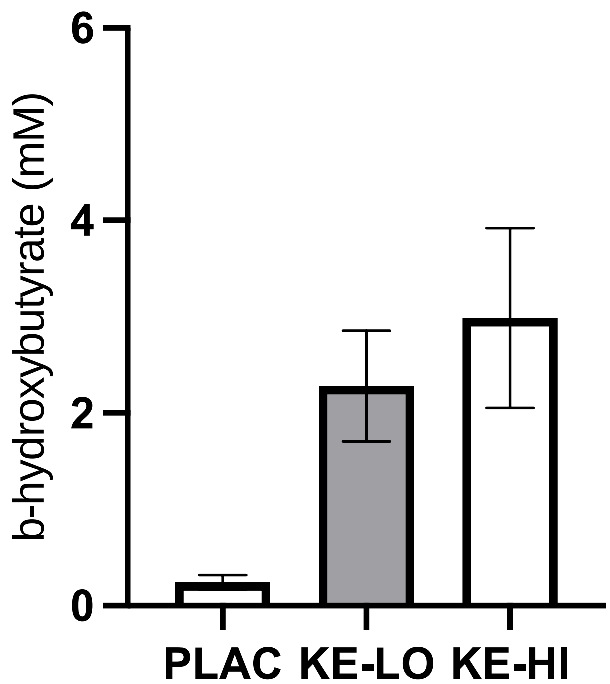


Figure 5: Venous β-hydroxybutyrate measured at rest following ingestion of a 0.3 or 0.6 g/kg body mass bolus of KE or a flavour-matched placebo. Data are mean±SD (n=26). *p<0.05 vs PLAC; +p<0.05 vs KE-LO

	PLAC	**KE-LO**	**KE-HI**
Glucose (mM)‡	5.7 (5.4-5.9)	4.8 (4.5-5.3) *	4.6 (4.2-5.1) *
Lactate (mM)	1.4 (1.1-2.0)	1.4 (1.0-2.3)	1.4 (1.1-2.3)
pH‡	7.351 (7.333-7.366)	7.344 (7.316-7.360)	7.333 (7.302-7.347) *
Bicarbonate (mM) ‡	29.3 ± 2.0	27.0 ± 2.3*	26.1 ± 2.3*
Total CO_2 (mmHg)‡	53.6 ± 5.5	50.5 ± 6.7*	50.0 ± 6.5*
Total O_2 (mmHg)	21.4 (17.3-25.5)	23.6 (19.7-28.4)	26.2 (21.2-33.9)

Table 2: Blood metabolite and gas data. Data are presented as mean±SD for n = 26 for bicarbonate and total CO_2, median (interquartile range) for all others. d_z: Cohen's effect size. ‡p<0.05 main effect of condition; *p<0.05 vs PLAC.

Submaximal Exercise Data

Exercise economy (p=0.23) and mean $\dot{V}O_2$ (p=0.21) were not different between conditions during submaximal exercise (Figure 6a and 6b, respectively). Exploratory

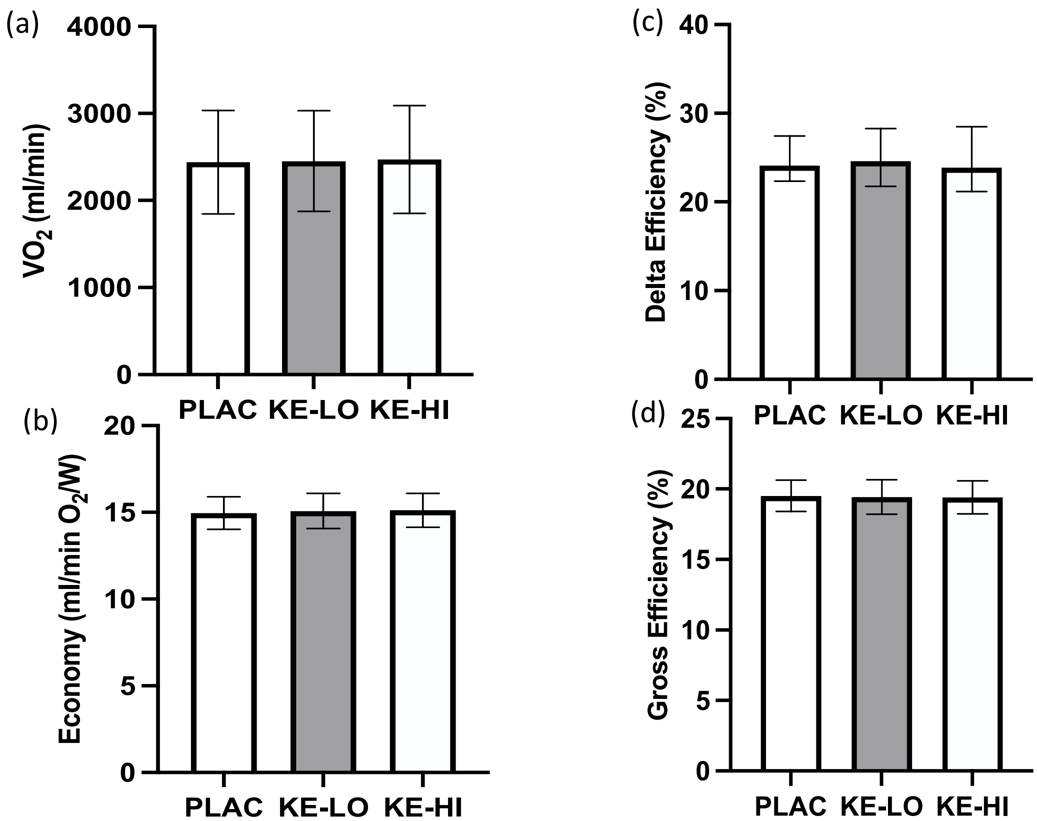


Figure 6: (a) Mean exercise $\dot{V}O_2$, (b) exercise economy, (c) delta efficiency, and (d) gross efficiency during the 3-stage, 15-min period of submaximal exercise after ingestion of a 0.3 or 0.6 g/kg body mass bolus of KE or a flavour-matched placebo. Data are mean±SD (n=28) for $\dot{V}O_2$, economy, and gross efficiency, and median (interquartile range) for delta efficiency.

analyses of steady state $\dot{V}O_2$ using the final 2 min of each stage also showed no difference in either mean $\dot{V}O_2$ or economy (p=0.15 and p=0.25, respectively). Similarly, there were no differences between condition for gross efficiency (p=0.54) or delta efficiency (p=0.37) efficiency (Figure 6c and 6d, respectively). Mean VCO_2 was not different between conditions (p=0.38). HRmean and V_E were both different (p=0.002 and p=<0.0001, respectively) such that they were lower in PLAC compared to both KE conditions, at 5[3-7] beats/min (p=0.0002, d_z= 0.90) and 3.0 [1.7-4.3] L/min (p=0.0003, d_z=0.62) lower vs KE-HI as well as 4 [1-7] beats/min (p=0.037, d_z=0.48) and 2.0 [1.0-3.0] L/min (p=0.01, d_z=0.85) lower vs KE-LO (Figure 7). RER was different between treatments (p=0.02) and 0.019[0.006-0.032] lower in KE-HI vs PLAC only (p=0.02, d_z=0.53; Figure 7c). There was no difference in RPE between conditions (12.6±0.9, 12.5±1.0, and 12.7±0.7 for PLAC, KE-LO, and KI-HI, respectively p=0.57). There were

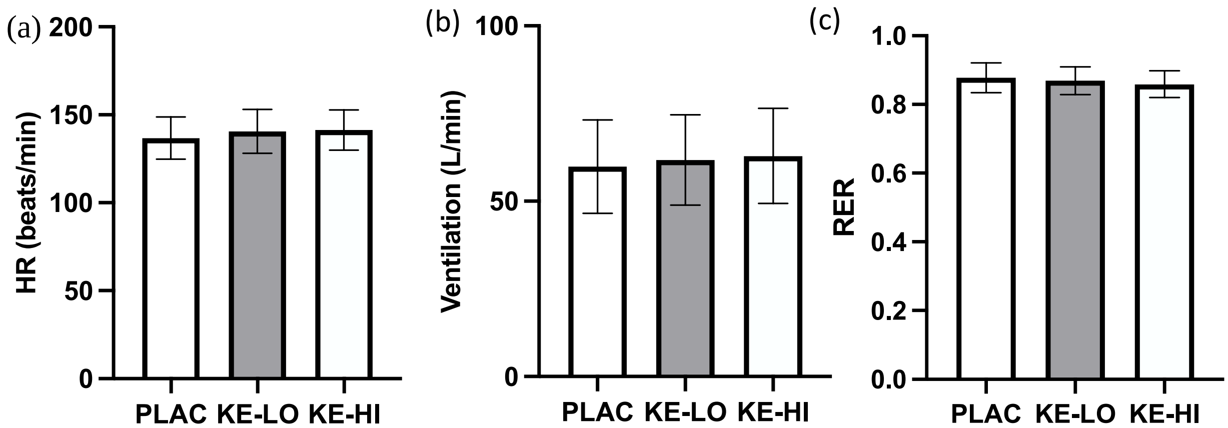


Figure 7: (a) Heart rate (HR), (b) ventilation, and (c) RER during the 3-stage, 15 min submaximal exercise bout after ingestion of a 0.3 g/kg or 0.6 g/kg body mass bolus of KE or a flavour-matched placebo. Bars are mean±SD (n = 28). *p<0.05 vs PLAC.

no interactions between supplement condition and exercise intensity for any variable (all $p>0.05$; Table 3). All submaximal exercise data are compiled in Table 4.

Table 3: Mean V̇O2, VE, and HR for each individual exercise intensity of the submaximal protocol. VT_1: first ventilatory threshold $\dot{V}O_2$: oxygen uptake; V_E: ventilation; HRmean: mean heart rate.

	75% VT_1			**100% VT_1**			**125% VT_1**		
	PLAC	**KE-LO**	**KE-HI**	**PLAC**	**KE-LO**	**KE-HI**	**PLAC**	**KE-LO**	**KE-HI**
Mean $\dot{V}O_2$ (L/min)	2.0 ± 0.4	2.0 ± 0.4	2.0 ± 0.5	2.5 ± 0.6	2.5 ± 0.6	2.5 ± 0.6	2.9 ± 0.7	2.9 ± 0.7	3.0 ± 0.8
Mean V_E (L/min)	45.5 ± 9.3	47.1 ± 8.4	47.5 ± 9.5	59.7 ± 13.3	61.5 ± 12.2	62.6 ± 13.1	74.5 ± 17.7	77.0 ± 17.9	78.6 ± 18.4
HRmean (beats·min^{-1})	119 ± 12	124 ± 13	124 ± 11	137 ± 13	141 ± 14	142 ± 12	152 ± 13	156 ± 13	156 ± 12

Table 4: Cardiorespiratory, power, and efficiency data during the 3-stage 15 min submaximal exercise protocol in PLAC, KE-LO and KE-HI.

	PLAC	**KE-LO**	**KE-HI**
$\dot{V}O_2$ (L/min)	2.4 ± 0.6	2.5 ± 0.6	2.5 ± 0.6
VCO_2 (L/min)	2.2 ± 0.5	2.2 ± 0.5	2.1 ± 0.5
RER‡	0.88 ± 0.04	0.87 ± 0.04	0.86 ± 0.04*
Ventilation (L/min)‡	60 ± 13	62 ± 13*	63 ± 14*
Mean heart rate (beats·min^{-1})‡	137 ± 12	141 ± 12*	141 ± 11*
Peak heart rate (beats·min^{-1})	158 ± 13	160 ± 13	160 ± 12
RPE	13 ± 1.0	13 ± 0.8	13 ± 0.7
Mean power output (W)	164 ± 43	164 ± 43	164 ± 43
Economy (ml O_2·W)	15.0 ± 0.9	15.1 ± 1.0	15.1 ± 1.0
Gross efficiency (%)	19.5 ± 1.1	19.4 ± 1.2	19.4 ± 1.2
Delta efficiency (%)	24.1 (22.4-27.3)	24.6 (21.9-28.1)	23.9 (21.6-28.4)
Steady State $\dot{V}O_2$ (L/min)	2.5 ± 0.2	2.6 ± 0.2	2.6 ± 0.2

Note: Data are presented as mean±SD for all data (n = 28) except delta efficiency, which is presented as median (interquartile range) for n = 28. $\dot{V}O_2$: oxygen uptake; VCO_2: carbon dioxide expelled; RER: respiratory exchange ratio; RPE: rating of perceived exertion; d_z: Cohen's effect size. PLAC: placebo; KE-LO: 0.3 g/kg dose of ketone monoester; KE-HI: 0.6 g/kg dose of ketone monoester. $^{‡}p<0.05$ main effect of condition; $^{*}p<0.05$ vs PLAC.

Peak Exercise Data

$\dot{V}O_2$peak at the end of the ramp test in each treatment was not different (3.97±0.84, 3.97±0.86, and 3.91±0.88 L/min for PLAC, KE-LO, and KI-HI, respectively p=0.11), and was equal to 97%, 97%, and 96% of baseline $\dot{V}O_2$peak for PLAC, KE-LO, and KE-HI respectively. Peak power output was different between treatments (p<0.0001), such that it was 10[4-14] W lower in KE-HI vs KE-LO (p=0.0013, d_z=0.76) and 12[7-16] W lower vs PLAC (p<0.0001, d_z=0.96; Figure 8a). Peak RER was 0.04[0.02-0.07] lower in KE-HI compared to PLAC (p=0.0002, d_z=0.89) and 0.03[0.01-0.06] lower in KE-LO (p=0.002, d_z=0.75; Figure 8b). HRpeak was not different between conditions 181±11, 181±12, and 180±11 beats/min for PLAC, KE-LO, and KI-HI, respectively p=0.09). Peak VCO_2 was lower in KE-HI compared to PLAC (p=0.0003, d_z =0.84) and KE-LO (p=0.01, d_z =0.67). All peak exercise data are compiled in Table 5.

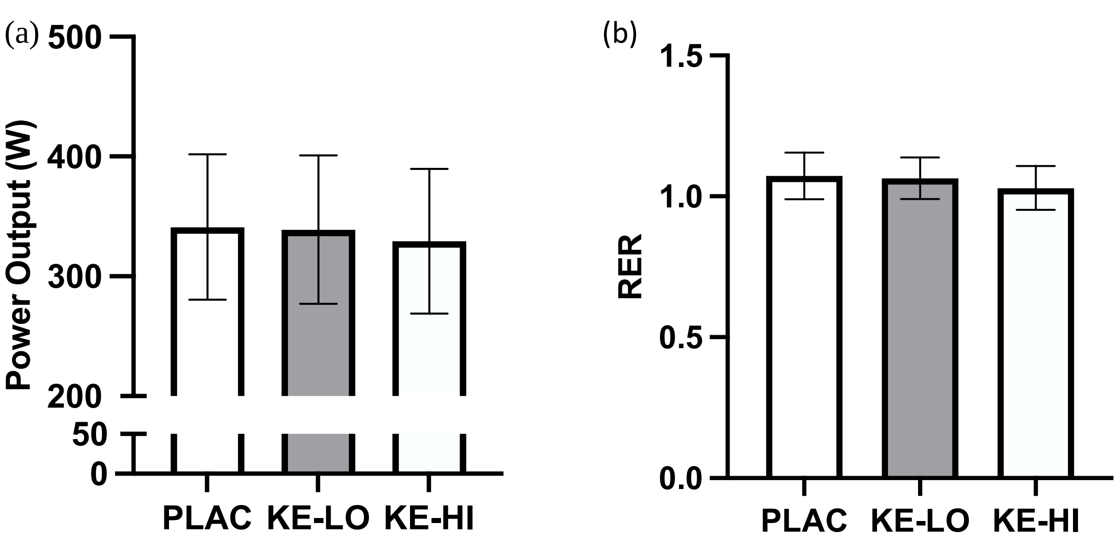


Figure 8: (a) Peak power output at the end of the ramp to exhaustion; (b) RER at $\dot{V}O_2$peak in PLAC, KE-LO, and KE-HI. Bars are mean±SD (n = 28). *p<0.05 vs PLAC; +p<0.05 vs KE-LO.

Table 5: Cardiorespiratory, power, and economy data during the ramp to volitional exhaustion in PLAC, KE-LO and KE-HI.

	PLAC	**KE-LO**	**KE-HI**
$\dot{V}O_2$ (L/min)	4.0 ± 0.8	4.0 ± 0.9	3.9 ± 0.9
$\dot{V}CO_2$ (L/min)‡	4.2 (3.5-4.8)	4.2 (3.3-5.0)	4.1 (3.2-4.6)*†
RER‡	1.07 ± 0.08	1.06 ± 0.07	1.03 ± 0.08*†
Ventilation (L/min)‡	136 ± 32	138 ± 32	133 ± 32
Mean heart rate (beats·min^{-1})	168 ± 12	170 ± 12	169 ± 11
Peak heart rate (beats·min^{-1})	181 ± 11	181 ± 12	180 ± 11
Peak power output (W)‡	341 ± 61	339 ± 62	329 ± 60*†

Note: Data are presented as mean±SD for all data (n = 28) except $\dot{V}CO_2$, which are presented as median (interquartile range) for n = 28. $\dot{V}O_2$: oxygen uptake; $\dot{V}CO_2$: carbon dioxide expelled; RER: respiratory exchange ratio; ‡p<0.05 main effect of condition; *p<0.05 different vs PLAC; †p<0.05 vs KE-LO.

Gastrointestinal Distress

There was a main effect of condition in total GI stress (p=0.026), with the mean score for KE-HI being greater than that of PLAC (0.43 vs 0.18, p=0.03) and KE-LO (0.43 vs 0.19, p=0.017). Further analyses revealed this was due to increased upper GI stress (p=0.016), with a higher mean score in KE-HI compared to PLAC only (0.76 vs 0.24, p=0.038). There was no effect of condition for lower or other GI stress (p=0.28 and p=0.065, respectively). Main effects of condition were found for belching (p=0.35, no differences between groups), bloating (p=0.024) and urge to regurgitate (p=0.013).

DISCUSSION

The primary novel finding of this study was that acute ingestion of a 0.3 or 0.6 g/kg body mass dose of a KE supplement did not alter exercise economy as compared to a flavour-matched placebo during submaximal exercise. We also found no differences between conditions in delta or gross efficiency. HR and V_E during submaximal exercise were higher in both KE-supplemented conditions compared to PLAC. The latter observations replicate findings from our previous work (McCarthy et al, 2021; McCarthy et al, 2023a) and are indicative of increased cardiorespiratory stress during submaximal exercise after acute KE ingestion. Peak power output achieved at $\dot{V}O_2$peak at the end of the ramp to exhaustion was lower in the KE-HI condition compared to PLAC, which also replicates a previous finding from our laboratory (McCarthy et al, 2021) and is suggestive of impaired high-intensity exercise capacity after KE ingestion.

Exercise efficiency and economy

The present study constitutes the most rigorous assessment to date of the effects of KE ingestion on exercise efficiency, in part owing to the relatively large sample size in comparison to previous work. To our knowledge, only one other study has specifically explored the effects of KE ingestion on exercise efficiency. Dearlove et al. (2021) reported a 7% improvement in delta efficiency in trained cyclists after the ingestion of a 0.25 g/kg bolus dose of KE compared to placebo, but no effect of a higher (0.75 g/kg) dose. In contrast, the present study found no effect of acute KE ingestion on delta efficiency, gross efficiency, or exercise economy after ingestion of either a 0.3 or 0.6 g/kg dose of KE compared to placebo. The reason for the equivocal findings between studies is unclear. The study by Dearlove et al. (2021) was based on a sample size of six participants, and the reported significant increase in delta efficiency was based on an exploratory analysis, with no main effect of condition being found ($p=0.098$). In contrast, the present study involved a total a sample size of 28, which was estimated based on a power analysis to detect a medium effect size, and we found no effect of condition on any measure of exercise efficiency. We therefore conclude that any potential influence of KE on exercise efficiency is small and unlikely to have any beneficial effect on exercise capacity.

Most studies including the present work have reported no effect of KE ingestion on exercise $\dot{V}O_2$ (Evans et al, 2019; Prins et al, 2020; Dearlove et al, 2021; McCarthy et al, 2021). Despite the primary finding of no difference in the various measures exercise efficiency or economy, there may have been subtle and potentially offsetting influences of KE ingestion on exercise $\dot{V}O_2$ but no measurable net change. That is, some mechanisms may have potentially increased $\dot{V}O_2$, and these were counteracted by others that reduced $\dot{V}O_2$. The proposed mechanism for why KE ingestion might enhance exercise efficiency through decreased $\dot{V}O_2$ centres on the oxidation of βHB resulting in a greater Gibbs free energy change as compared to pyruvate and glucose (Sato et al, 1995; Veech et al, 2004). βHB oxidation also has a higher ratio of ATP produced per mol of O_2 used (i.e. P/O ratio) when compared to fatty acids (Karwi & Lopaschuk, 2021). One commonly cited study used as an example for the energetic efficiency of KBs showed a ~25% improvement in the hydraulic efficiency of a working rat heart after KBs were added to it (Sato et al, 1995). Most work pertaining to the energetic efficiency of KBs examine myocardial substrate metabolism, and as different tissues possess different capacities to use KBs, potential differences in efficiency are not necessarily applicable to skeletal muscle. However, it stands to reason that the energetic efficiency associated with KB oxidation would be reflected in a lower level of oxygen consumption at a fixed work rate (Dearlove et al, 2021; Evans et al, 2022), although this was not observed in the present study. KBs have minimal ability to drive cellular respiration alone, decreased further in the presence of pyruvate, which outcompetes KBs by up to threefold (Petrick et al, 2020).

KE ingestion has also been reported to increase submaximal $\dot{V}O_2$ at a fixed workload compared to control (Poffé et al, 2021a), which is suggestive of impaired exercise efficiency. There are several mechanisms by which this may occur, potentially offsetting any reductions in $\dot{V}O_2$. Firstly, the P/O ratio of βHB (~2.5 ATP/O_2), while higher than that of fatty acids (2.33 ATP/O_2), remains lower than that of glucose (~2.58 ATP/O_2). Therefore, should βHB replace glucose as an energy substrate during exercise, O_2 consumption would be higher. Nutritional ketosis also may induce a glycogen 'sparing' effect and lowers blood glucose, further reducing the contribution of CHO to energy expenditure and increasing exercise $\dot{V}O_2$. Such changes would be reflected in a lower exercise RER, which is seen in the present work in the KE-HI condition (Table 4). Furthermore, the increase in ventilation found in this and other works (McCarthy et al, 2021; Poffé et al, 2021a; McCarthy et al, 2023a) demonstrates an increase in the work of breathing. This places greater stress on the respiratory musculature such as the diaphragm, resulting in more O_2 being required to meet the demands of the increased work, and therefore increasing $\dot{V}O_2$. Exogenous KE supplementation has also been found to increase the contribution of fatty acids to energy provision during moderate intensity exercise (Cox et al, 2016), which would reduce exercise efficiency through increased $\dot{V}O_2$ due to their lower P/O ratio compared to glucose (Karwi & Lopaschuk, 2022).

With such mechanisms existing that may cause both increases and decreases in exercise $\dot{V}O_2$, the lack of effect on exercise efficiency seen in the present study may be due to an 'offsetting' effect. $\dot{V}O_2$ may be being increased through some mechanisms and decreased through others, effectively cancelling one another other out and causing the lack of effect we see in the present study. A pertinent point is that, due to the minor contribution of KBs to energy expenditure at ≤5% (Evans et al, 2017; Evans et al, 2022), it is questionable whether such a small contribution to energy expenditure would have any meaningful effect on $\dot{V}O_2$, and therefore exercise efficiency, in the first place. Furthermore, KBs have been shown to minimally drive mitochondrial respiration on the presence of pyruvate (Petrick et al, 2020), further calling into question their influence on exercise efficiency. Future research should delve further into these proposed mechanisms to elucidate if there is indeed an 'offsetting' effect caused by multiple physiological mechanisms working against one another.

Cardiorespiratory exercise responses

Acute KE ingestion increased HR during submaximal exercise compared to control, replicating results found from others including own laboratory (Robberechts et al, 2022; McCarthy et al, 2021; McCarthy et al, 2023a). This effect seemed to be independent of the size of the KE dose, suggesting that a ceiling of ketosis exists at which

HR is raised but any increase in blood [βHB] beyond that does not result in any further increases. Additionally, the increases were consistent for each exercise intensity (Table 3). However, other works have found no changes in exercise HR (Evans & Egan, 2018; Dearlove et al, 2021; Poffé et al, 2021a; Poffé et al, 2021b). The precise mechanisms responsible for the KE-associated increase in HR are unclear, but possible explanations include the influence of increased blood [norepinephrine] (Robberechts et al, 2022), and nicotinic acid receptor activation (Gadegbeku et al, 2003; Newman & Verdin, 2014).

Submaximal ventilation was increased with KE supplementation, similar to what was found in previous work from ours and other labs (Poffé et al, 2021a; McCarthy et al, 2021; McCarthy et al, 2023a). We suggested that this KE-associated increase in ventilation is related in part due to blood acidosis (McCarthy et al, 2023a), which the current study supports to some extent with a decrease in blood pH being seen in the KE-HI condition. However, the same change in pH was not seen in KE-LO despite increase V_E, and no correlation between the KE-associated change in pH and the changes in V_E was found. Therefore, the effect of KE on submaximal exercise V_E seems to be somewhat affected by blood pH, but also involves other, yet unknown factors.

Peak exercise responses

It has been proposed that an ergogenic effect of KE supplementation may be seen if circulating blood βHB levels reach ~2 mM (Evans et al, 2017). While the present experiment was not specifically designed assess this issue, peak power output achieved at the end of the ramp provides an index of the capacity for high-intensity exercise performance. However, no improvements in peak power output were seen in KE-LO, where pre-exercise βHB concentration for was 2.3 mM, therefore not supporting the notion of this 'ergogenic threshold'. An ergolytic effect was seen in the KE-HI condition where pre-exercise βHB reached ~3.0 mM however, finding a 12 W decrease in peak power output which agrees with previously reported data using an identical KE dose as KE-HI (McCarthy et al, 2023a). Despite this lower power output, none of $\dot{V}O_2$peak, HRpeak, or RPE were statistically different in KE-HI compared to baseline or PLAC, so the change is likely not attributable to participants ceasing the test prior to reaching maximal effort. While the body of work surrounding the effects of KE ingestion on exercise performance are limited, these and other data (Leckey et al, 2017; Poffé et al, 2021b; McCarthy et al, 2023b) support the notion that KE supplementation impairs short-duration, high-intensity exercise.

Nutritional ketosis induced through low-CHO/high-fat (LCHF) dietary interventions suggest that high-intensity exercise performance may be affected by an impairment of CHO utilization (Burke et al, 2017; Burke, 2021). LCHF diets have also been reported to impair exercise economy on account of increased fat oxidation, which of course did not occur in the present work, although KE supplementation can increase the contribution of fatty acids to energy provision as well (Cox et al, 2016). KBs may also inhibit the activity of key metabolic enzymes associated with glycolysis such as phosphofructokinase (PFK) and pyruvate dehydrogenase (PDH) (Maizels et al, 1977; Laughlin et al, 1994). While speculative, decreased CHO utilization could be reflected by lower pre-exercise blood [glucose] (Table 2) and exercise RER (Table 4), both of which were observed in the present experiment. However, with the likely very small contribution of βHB towards total energy expended, it is unlikely to be sufficient to cause meaningful shifts in CHO metabolism such that peak work capacity is reduced. Further work is needed to explore the influence of KE supplementation on substrate oxidation and its influence on exercise performance, as well as the potential of a dose-response relationship existing with respect to blood βHB concentration and exercise performance.

Strengths and Limitations

A strength of the present work was our robust final sample size, which exceeded that of comparable previous studies and was based on an explicit estimate to detect a change in exercise economy with a medium effect size. The study protocol was also registered prior to data collection and efforts were made to reduce the potential for bias. These included having separate, unblinded researchers randomize and counterbalance the supplements, as well as analyze and record blood sample data while not interacting with participants. The principal researcher remained blinded throughout the statistical analysis of all data save that of blood samples. A potential limitation of this study exists is the usage of exercise stages at 5 min of length and the influence of the $\dot{V}O_2$ slow component on submaximal $\dot{V}O_2$ and economy at exercise intensities at or above VT_1. While statistical analyses were performed using expired gases from the last 2 min of each stage (i.e., after steady state was likely reached) showed that there remained no difference between any conditions, it is possible that the influence of the $\dot{V}O_2$ slow component affected $\dot{V}O_2$ at the later exercise stages of 100% and 125% VT_1, either causing steady state to be reached later or potentially not at all. However, this author argues that measuring mean submaximal $\dot{V}O_2$ throughout the whole 15 min exercise bout takes all potential influencing factors into account for each participant, effectively eliminating any influence of the slow component.

Conclusion

Neither the ingestion of a 0.3 g/kg or 0.6 g/kg body mass bolus dose of KE altered exercise economy or exercise efficiency during submaximal cycling compared to placebo. This result may not be due to KE ingestion having no effect on exercise efficiency *per* se, but rather may be the result of a $\dot{V}O_2$ 'offsetting' effect, in which KE ingestion reduces it via some mechanisms but improves it through others. Our findings agreed with those from our previous work in that pre-exercise KE ingestion increased both HR and V_E during submaximal exercise and decreased peak work capacity through a reduction of peak power output at $\dot{V}O_2$peak. Given the absence of a change in exercise efficiency or economy, the precise mechanisms for this reduction are unclear and should be explored in further depth, but it may have been through an impairment of CHO utilization as reflected by reduced exercise RER and pre-exercise blood glucose. Researchers should continue to explore the influence of ketone body supplementation on exercise metabolism with respect to mechanisms affecting exercise $\dot{V}O_2$, as well as their effects on high intensity exercise performance.

Appendix A: HiREB study approval letter

HiREB

Hamilton Integrated Research Ethics Board

Nov-10-2022

Project Number: 15110

Project Title: The Effects of Acute Ketone Monoester Supplementation on Exercise Efficiency and the Influence of Dose and Intensity

Principal Investigator: Dr Martin Gibala

The Hamilton Integrated Research Ethics Board (HiREB) has reviewed and approved the abovementioned study.

The following documents have been approved on both ethical and scientific grounds:

Document Name	Document Date	Document Version
Bone, J - Data Collection Sheet Trials	Jul-12-2022	v1
GI and blinding questionnaires	Jul-12-2022	v1
Bone, J - 24 h Dietary recall	Jul-12-2022	v1
Bone, J - Baseline Collection Sheet	Jul-12-2022	v1
Bone, J - Blood Analyses Data Collection Sheet	Jul-12-2022	v1
Bone, J - Recruitment poster V2 - Clean	Sep-21-2022	v2
Bone, J - Email Correspondence V2 - Clean	Sep-21-2022	v2
Bone, J - Phone Script KEF	Sep-21-2022	v1
Gibala Lab Menstrual Cycle Questionnaire	Sep-21-2022	v1
Bone, J - Participant Intake Questionnaire (KEF)	Sep-21-2022	v1
Bone, J - Participant Information Sheet V2 - Clean	Sep-21-2022	v2
Bone, J - Study Protocol V2 - Clean	Sep-22-2022	v2
Bone, J - Consent Form V2 - Clean	Sep-22-2022	v2

The following documents have been acknowledged:

Document Name	Document Date	Document Version
CITI Program Refresher 2021-08-23 ID 40783773	Jul-11-2022	1
KEF Response Letter - FINAL	Sep-21-2022	v1
KEF R2 Response Letter - FINAL	Nov-10-2022	v1

In light of the current COVID-19 pandemic, while HiREB has reviewed and approved this application, the research must be conducted in accordance with institutional and/or public health requirements.

Please Note: All consent forms and recruitment materials used in this study must be copies of the above referenced documents.

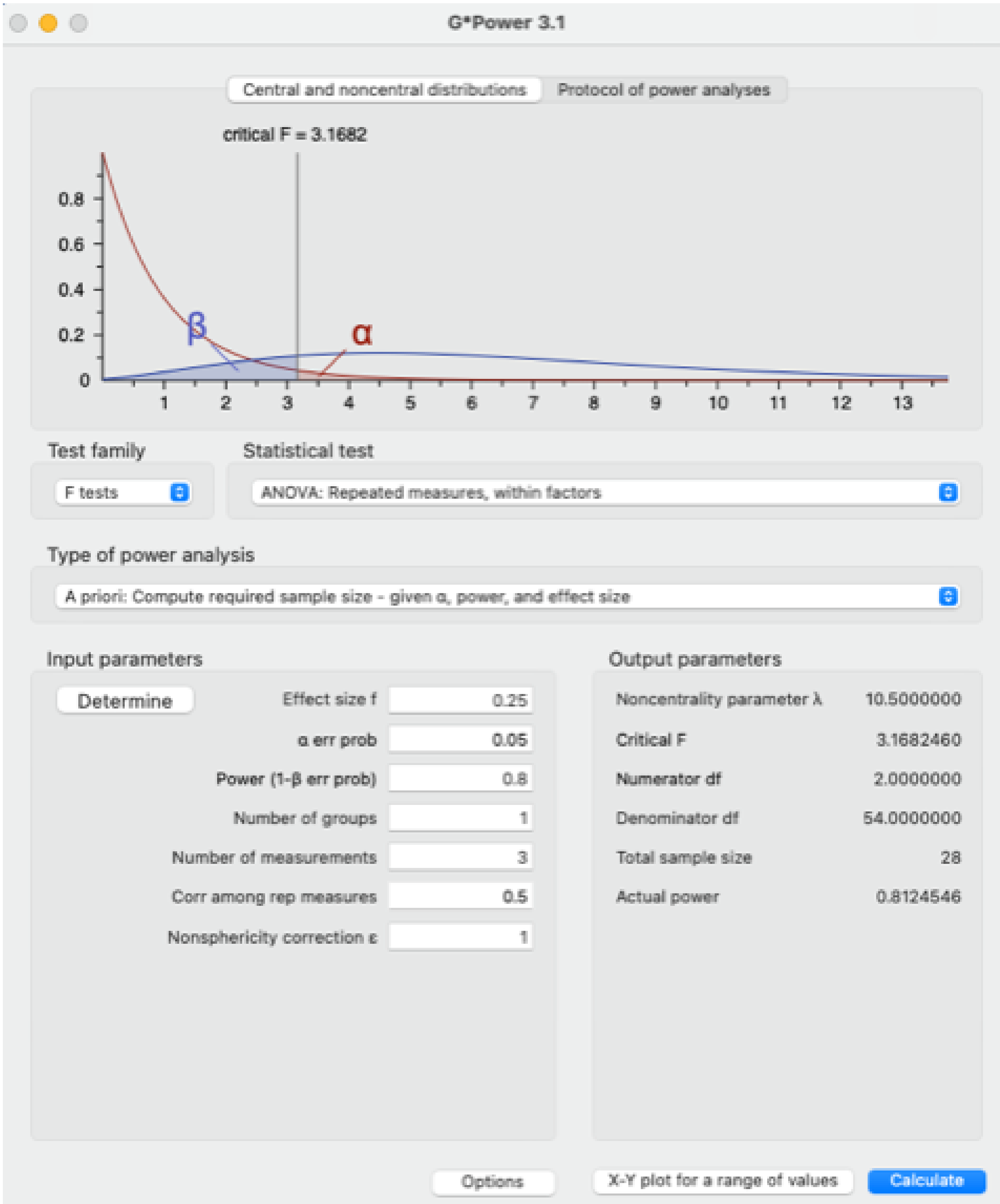
We are pleased to issue final approval for the above-named study for a period of 12 months from the date of the HiREB meeting on August 3, 2022. Continuation beyond that date will require further review and renewal of HiREB approval. Any changes or revisions to the original submission must be submitted on a HiREB amendment form for review and approval by the Hamilton Integrated Research Ethics Board.

PLEASE QUOTE THE ABOVE REFERENCED PROJECT NUMBER ON ALL FUTURE CORRESPONDENCE

Sincerely,

Dr. Frederick A. Spencer, MD

Appendix B: G*Power analysis to determine sample size



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