MEASUREMENT OF BREATH ACETONE IN THE DETECTION OF LOW CARBOHYDRATE AVAILABILITY FOLLOWING IMPLEMENTATION OF "TRAIN LOW" STRATEGIES

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MATERIAL ABSTRACT

Enhancing the metabolism of athletes can be achieved through the manipulation of nutrition and exercise variables. Muscle biopsies are currently viewed as the 'gold-standard' for assessing muscle glycogen levels of athletes. However, the process of obtaining a muscle biopsy is invasive and takes a considerable time to analyse. Following research that has implemented muscle biopsies, it has been suggested that an acute reduced carbohydrate intake in conjunction with a suitable training intensity (known as "training low") has shown to elicit conducive metabolic adaptations to increase the sustainability and production of adenosine triphosphate (ATP) to support endurance athletes. The overarching purpose of this research was to establish and refine a suitable protocol, which could be employed to analyse the effectiveness of a number of "train low" strategies at eliciting low carbohydrate availability in endurance athletes. Six key aims were identified to guide the research, which focused on the integration of chemistry and sports science equipment, identifying the most effective sequence to collect the measurements required, and comparing and understanding two breath acetone (BrAce) detectors for assessing carbohydrate availability. This thesis presented a methodological-development study to address the purpose and aims through a series of four pilot studies. The two BrAce detectors included a Cavity Enhanced Laser Induced Fluorescence (CELIF) prototype (made by the Chemistry Department at Durham University) and a Ketonix® BrAce detector, which is currently in the breath analyser market. In addition to the BrAce detectors, other metabolic measurements included capillary blood glucose, betahydroxybutyrate and gas analysis (i.e., respiratory exchange ratio). The pilot studies concluded with an experimental pilot, which involved an endurance athlete (male, ultra-endurance, running; age: 38 years; stature: 1.76 m; body mass: 66.1 kg), who followed the agreed protocol. Taking the range of measurements sequentially rather than in parallel

was the most effective, with the CELIF BrAce concentrations reflecting the values of the accompanying metabolic measures more than the Ketonix®, with the Ketonix® providing a wider variance of measurements within and between participants. The participant in the experimental trial was unable to complete the exercise protocol after following the “train low” strategies. Overall, the agreed protocol worked effectively, with some revaluation required for elements of the equipment and protocol. The CELIF BrAce detector provided an indication that it detects carbohydrate availability more effectively than the Ketonix®. However, further refining and development is required to improve the prototype’s effectiveness of detecting low carbohydrate availability.

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CHAPTER 1 – INTRODUCTION

1.1 Background to exercise metabolism

This chapter presents an introduction to exercise metabolism and how exercise metabolism can be monitored. Exercise metabolism refers to how an individual utilises the food they consume and transfer the food into energy during exercise (Cox and Clarke, 2014; Impey *et al.*, 2018; Jeukendrup and Gleeson, 2019; Burke, 2021). The energy currency of living organisms is ATP, which can be generated in different ways (Mougios, 2020). Effective metabolism during exercise is characterised by the transfer of sufficient quantities of ATP to continue exercise at a given intensity, whilst being able to spare adequate stores of ATP for the latter stages of exercise to allow an individual to continue exercising at the required intensity (Burke *et al.*, 2011; Bartlett *et al.*, 2013; Hawley and Morton, 2014; Mougios, 2020). In short, an individual's exercise metabolism capacity is determined by the level of production and sustainability of ATP during exercise. Despite the concept of exercise metabolism being unanimous across previous centuries there was (and continues to be) scientific debate surrounding how to optimise and monitor an individual's metabolism during exercise (Hawley *et al.*, 2015). Debated topics included the sources of energy for muscular activity, exercise and nutrition manipulations to alter patterns of fuel utilisation, factors limiting physical work capacity and the most effective way to monitor changes in metabolism (Hawley *et al.*, 2015; Mougios, 2020). These considerations highlight that although research has established what exercise metabolism is, the specificity of variables in which are known to be involved in enhancing exercise metabolism are not completely understood.

It is well established that carbohydrate and fat are the main sources of fuel that athletes use during exercise, with proteins providing minimal amounts of energy. Within the muscle,

carbohydrate is stored as glycogen and is broken down into glucose (Mougios, 2020). Glucose is the main source of fuel for high intensity exercise because it is more readily converted to ATP in comparison to fat (Alberts *et al.,* 2002; Mougios,2020). Glycogen depletion, the exhausting of the muscles' glycogen stores, can occur intentionally and unintentionally and through the manipulation of carbohydrate intake and / or exercise (Burke *et al.,* 2011; Bartlett *et al.,* 2015; Jeukendrup and Gleeson, 2018). Intentional glycogen depletion is often used when an individual wants to "train low" (i.e., train with a reduced carbohydrate intake) for the purposes of eliciting conducive physiological adaptations to increase the body's capacity to metabolise fat, which is a virtually limitless store of energy (Bartlett *et al.,* 2015; Impey et al., 2018). In summary, through manipulating nutrition and exercise variables, an individual's metabolism during exercise can be enhanced.

1.2 Rationale

To ascertain a true measurement of muscle glycogen levels, a muscle sample must be obtained from a biopsy. This invasive procedure requires a trained individual to take the biopsy and laboratory-based equipment for processing and analysis, which takes a considerable amount of time (Burke *et al.,* 2011). Typically, glycogen depletion strategies are very strenuous to ensure that carbohydrate availability, the level of carbohydrate available for the body to utilise have been depleted (Burke, 2010; Jeukendrup and Gleeson, 2018). When incorporating "train low" sessions the aim is to be reliant on fat as the primary fuel rather than carbohydrate (Bartlett *et al.,* 2013; Hawley and Morton, 2014). Measuring the RER, which is the volume of oxygen consumed ($\dot{V}O_2$) divided by the volume of carbon dioxide produced ($\dot{V}CO_2$) in expelled air can indicate whether an individual is burning predominantly carbohydrate or fat (Krogh, 1920). However, using RER does not have the resolution that would allow someone to pinpoint when they are burning predominantly fat. The more

accurately this point can be determined, the more nuanced the strategies can be that result in a "train low" state (Hawley and Morton, 2014). This is important because if an individual is restricting their energy or carbohydrate intake to a greater extent than is required to achieve a "train low" status, this could have an impact on their health (Burke *et al.*, 2011). Insufficient intake, whether energy or carbohydrate, suppresses an individual's immune system, impairs training adaptations and negatively impacts on recovery (Baar and McGee, 2008; Burke *et al.*, 2011; Mata *et al.*, 2019). Furthermore, unintentional glycogen depletion may occur when an individual trains and does not adequately replace the glycogen they use, which could be due to restricted intake or a lack of understanding of the amounts of carbohydrate that should be consumed (Burke *et al.*, 2011). This situation can also be observed in injured individuals (innocently causing delayed healing and recovery and possibly further injury by trying to avoid unwanted weight gain by undernourishment), those that are trying to lose weight and those that suffer from Relative Energy Deficiency in Sports (RED-S) (Bartlett *et al.*, 2015; Dipla *et al.*, 2021). Consequently, having a real-time, sensitive measurement tool to monitor changes in metabolism would considerably decrease the 'guess work', and reduce the risk of negative consequences of manipulating carbohydrate intake and increasing the chances of experiencing the proposed physiological benefits.

1.3 Research purpose and aims

The overarching purpose of this methodological development study is to establish and refine a suitable protocol, which could be employed to analyse the effectiveness of different "train low" strategies at eliciting low carbohydrate availability in endurance athletes. Six key aims were identified to guide the research and are outlined below:

1. Establish the most efficient procedure for collecting the required gas samples.
2. Identify the most effective sequence to collect the measurements required.

3. Compare the BrAce concentrations measured by the CELIF BrAce prototype (Chemistry Department at Durham University), and a BrAce detector currently on the market (Ketonix®).
4. Understand the impact on the readings generated from adjusting the Ketonix® app settings.
5. Understand whether measuring BrAce is an effective method to detect low carbohydrate availability following the implementation of "train low" strategies.
6. Explore whether the agreed protocol for the experimental pilot study was practically effective with endurance athletes during exercise.

1.4 Thesis structure

Following this introduction, Chapter 2 is a literature review, which is followed by Chapters 3-5 that focus on the development of the methods. Specifically, Chapter 3 ascertains how the sports science and chemistry equipment can be integrated most effectively. Chapter 4 focuses on understanding the most practically effective protocol for the range of metabolic measurements that need to be taken. Chapter 5 compares the resting BrAce values of the CELIF BrAce prototype and the Ketonix® device after two non-endurance participants follow three nutrition strategies. The method development chapters (3-5) are then implemented in the experimental pilot study in Chapter 6, where an ultra-endurance participant completed a baseline testing session and four "train low" strategies. Following the experimental pilot study, an overall discussion in Chapter 7 reflects on the protocol, followed by limitations of the protocol and what the focus of the PhD will be. Chapter 8 concludes the thesis with the addition of an overall reflection of the research journey in Chapter 9.

CHAPTER 2 – LITERATURE REVIEW

2.1 Endurance exercise

Endurance sports or exercise require high muscular recruitment and aerobic fitness levels to work in unison (Cox and Clarke, 2014; Mwangi *et al.,* 2021; Rosenberg and Sailors, 2021). The physiological underpinnings of endurance sport contrast sports such as sprinting events (i.e., lasting < 10 s), which primarily rely on type I fast twitch fibres and anaerobic fitness (Spencer *et al.,* 2005). Saris et al. (2003; Burke, 2021) define endurance exercise as continuous events > 30 min with endurance events commonly lasting 3-5 h (e.g., triathlons, marathons), with some events lasting longer (≥ 12 h) (e.g., ironman, stage cycling, ultra-marathons). Due to endurance sport success (i.e., finishing the race in the shortest amount of time) being determined by the ability to sustain the highest power output for the event duration, endurance events involve a very high percentage of an individual's maximal aerobic intensity ($\dot{V}O_{2max}$) (Joyner and Coyle, 2008; Tucker, 2016; Rosenburg and Sailors, 2021). Moreover, key characteristics of elite endurance athletes include having a high peak aerobic capacity ($\dot{V}O2_{peak}$), high muscle oxidative capacity and high exercise economy (Joyner and Coyle, 2008). Furthermore, the demands of endurance sport induce mitochondrial and muscular training adaptations with the level and quality of adaptations accrued by genetics and training age (Holloszy, 1967; Tarnopolsky *et al.,* 2007; Bartlett *et al.,* 2013). Overall, there is a wide variety of endurance events, which are classified by the amount of time they take to complete, yet the physiological profile for success in endurance sport or exercise are suggested to be similar.

The main fuel sources used for energy during endurance exercise are carbohydrate and fat. The relative contribution of each substrate is dependent on exercise intensity, exercise duration and

athlete training status (Cermak and van Loon, 2013). Carbohydrate is a rapidly available but limited source of energy and is predominantly utilised during the onset of exercise and high-intensity activity (e.g., sprinting) (Saltin and Karlsson, 1971; Hawley and Morton, 2014). The respiratory quotient (RQ) of carbohydrate, the proportion of CO_2 volume generated to O_2 volume consumed at the cellular level, highlights how and when carbohydrate is metabolised. The RQ helps us understand how and when carbohydrate is metabolised, because the RQ for carbohydrate is ≥ 1.0 due to a higher volume of CO_2 being produced in comparison to O_2 (Jeukendrup and Gleeson, 2018). The increase in the CO_2 volume happens at higher intensities due to the cardiorespiratory strain, which happens when trying to maintain homeostasis, and when this strain occurs, carbohydrate is primarily metabolised (Kiens *et al.*, 1993). Carbohydrate is stored in the body predominantly as glycogen in muscle (300 g) and liver tissue (90 g) as well as the blood stream (30 g) (Jeukendrup and Gleeson, 2018; Bailey and Hennessy, 2020). This amounts to ~1700 kcal of available energy from carbohydrate at any one time without replenishing (Jeukendrup and Gleeson, 2018; Bailey and Hennessy, 2020). As a result, to maintain performance capacity, endurance athletes are required to replenish their glycogen stores every 1-3 h during competition due to some endurance events expending ~8000 kcal from athletes (Burke *et al.,* 2011; Hearris *et al.,* 2018; Burke, 2021). Through refuelling their carbohydrate stores, endurance athletes will be able to withstand more high intensity activity when their cells' RQ increases due to the increase in CO_2 (Burke *et al.,* 2021). So, due to carbohydrate being a limited source of energy, and not providing sufficient energy for endurance performance lasting > 1 h, refuelling of carbohydrate during events needs to be considered to support athletes during the high-intensity elements of their activity.

Conversely to carbohydrate, fat takes longer to metabolise and transfer to energy. When fat is transferred to energy it is a longer-lasting source of energy and is the primary fuel when

exercising at a lower intensity (highlighted by the RQ of fat = ~0.6) and/or when carbohydrate availability is limited (Burke *et al.*, 2011; Bartlett *et al.*, 2015; Sansone *et al.*, 2018). For healthy individuals, fat stores are virtually limitless, however, the inability of fat metabolism to support high-intensity efforts impairs endurance performance, creating a limitation to fat metabolism (Sansone *et al.*, 2018; Burke, 2021). The mechanism behind the inability of fat utilisation at high intensities is still not fully elucidated with scholars hypothesising a number of theories. To provide a couple of the most prominent theories within the literature, Horowitz and Klein (2000) suggested the fatty acids (FAs) within adipose tissues are 'trapped' due to a decrease in blood flow. Additionally, van Loon et al. (2001; Peric *et al.*, 2016) reported low-fat utilisation during high-intensity exercise, which was suggested to be because of a significant decrease in muscle free carnitine pool compared to values at rest. The decrease in muscle free carnitine is problematic for fat metabolism due to carnitine having the function of transferring long-chain FAs to the mitochondria for subsequent beta-oxidation to be converted into free fatty acids (FFAs) (Alberts *et al.*, 2002; Longo *et al.*, 2016). The van Loon and colleagues' study with eight male cyclists reported the decrease in the muscle free carnitine pool was stimulated by a significant ~three-fold increase in the muscle pyruvate dehydrogenase complex activation when comparing resting values to exercising at 75% of maximal power output (W_{max}) (0.57 ± 0.08 vs 1.67 ± 0.32 mmol·min^{-1}, $P < 0.05$). The protocol utilised by van Loon et al. (2001) reflected a similar nature to that of endurance exercise. The protocol was incremental over 90 min where the intensity started at 40% W_{max}, and increased to 55% W_{max} at 30 min, then to 75% W_{max} at 60 min until the completion of 90 min. Consequently, because of the protocol reflecting similar demands to that of endurance exercise, the results of this study are suitable to suggest that similar findings would be found with endurance athletes, whereby endurance athletes will have an increased reliance on carbohydrate rather than fat at high intensities. So, despite the theory

for understanding why fat metabolism is limited at high intensities being equivocal, the research agrees that the changes in physiology during high-intensity exercise in comparison to at rest or low-intensity exercise, leads athletes to prioritise carbohydrate as the primary fuel rather than fat.

From understanding the functions of carbohydrate and fat and the range of endurance events that there are, it is important to understand how each substrate is applied within the variety of endurance events. The literature has proposed in endurance events lasting 1-3 h (e.g., marathon run, skiing cross-country events), whereby athletes are exercising at a higher absolute intensity consistently (80-95% of $\dot{V}O_{2max}$) and are therefore utilising higher levels of muscle glycogen over a shorter period in comparison to ultra-endurance athletes (Burke, 2021). Due to the high demand of muscle glycogen, endurance athletes will need to replenish their muscle glycogen stores during a race if they do not want to inhibit their endurance capacity (Joyner *et al.,* 2011; Tucker, 2016; Burke, 2021). In contrast to the events lasting 1-3 h, i.e., longer events > 3 h (e.g., ironman triathlon, cycling stage races) rely on a lower 'background' intensity, with bursts of activity at or above critical velocity, thus having a higher reliance on fat than carbohydrate metabolism (Tucker, 2016; Burke, 2021). Even though endurance athletes competing in events > 3 h have a higher reliance on fat metabolism, these athletes would still need to replenish their muscle glycogen stores at points during a race, however, this would usually be later on in a race in comparison to endurance athletes who compete in shorter distance races. When and how many times endurance athletes competing in longer endurance events would need to refuel would be dependent on their pacing strategy (Jeukendrup and Gleeson, 2018; Burke, 2021). In summary, different endurance events rely on different proportions of carbohydrate and fat metabolism, which impacts their fuelling strategies during races.

2.2 Human metabolism

Anabolism and catabolism are the two phases of metabolism, with this subsection focusing on catabolism. The reason why this subsection will mainly focus on catabolism is because catabolism is the process when the food that humans eat is broken down into the human body's energy currency, ATP along catabolic pathways to allow muscle activity (i.e., contractions) to take place (Mougios, 2020). By way of comparison, anabolism is the alternative process, where simple molecules are 'built-up' to larger and more complex molecules (Mougios, 2020). Anabolism will be the main focus in the subsequent section.

2.2.1 Catabolism

Humans obtain energy by breaking down and utilising food. Carbohydrate, fat and protein (known as 'biomolecules' or 'macronutrients') are the three fundamental sources of energy that sustains reactions inside cells within the human body that allows humans to function (Jeukendrup and Gleeson, 2018). These life sustaining reactions generate and maintain the biological order that allows the human body to function (Alberts *et al.,* 2002). There are three stages by which humans extract energy from biomolecules (see Figure 1). In Stage 1 no ATP is produced, it is instead viewed as a preparatory stage for ATP to be subsequently synthesised (Mougios, 2020). During Stage 1, the macromolecules in food are broken down into their monomers through the actions of enzymes. Carbohydrate is converted into glucose and related compounds; lipids are converted into glycerol and FAs; and proteins are converted into amino acids (Mougios, 2020). These reactions in Stage 1 take place as part of the digestion process and then into the cytosol of the human cell where the small organic modules begin their gradual oxidation (Tarnopolsky *et al.,* 2007). Stages 2 and 3 occur solely within the cells, with the start of Stage 2 taking place within the cytosol and ending in the major energy-converting organelle,

the mitochondria (Alberts *et al.*, 2002). Finally, the whole process during Stage 3 takes place in the mitochondria (Alberts *et al.*, 2002; Murray and Rosenbloom, 2018).

During Stage 2, the products of Stage 1 are catabolised into simpler metabolites, which lie at the focal point of metabolism. Each molecule of glucose is degraded via glycolysis into two smaller molecules of pyruvate (Alberts *et al.*, 2002). The process of glycolysis also accommodates the production of glycerol from the breakdown of lipids (Cox and Clarke, 2014). During pyruvate formation, ATP and nicotinamide adenine dinucleotide (NADH, produced from NAD^+ during glycolysis), which are activated carrier molecules (molecules that stores easily-exchangeable energy) are produced (White and Schenk, 2012; Jeukendrup and Gleeson, 2018). After the pyruvate transports from the cytosol into the mitochondria, each pyruvate molecule is converted into CO_2 plus a two-carbon acetyl group, which subsequently becomes attached to coenzyme A, forming another activated carrier molecule acetyl CoA (Alberts *et al.*, 2002). Large amounts of acetyl CoA are co-produced by the breakdown and oxidation of FAs from fats, with the support of beta-oxidation (Cox and Clarke, 2014). The FAs are then transported into the blood stream and imported into the cells as FAs and then transferred into the mitochondria for acetyl CoA production.

For the final stage (Stage 3), the oxidative breakdown of food molecules takes place entirely in the mitochondria. The acetyl group in acetyl CoA is linked to coenzyme A though a high-energy linkage, thus making it easily transferable to other molecules (Alberts *et al.*, 2002; Jeukendrup and Gleeson, 2018). After its transfer to the oxaloacetate, the acetyl group is oxidised into CO_2 through the citric acid cycle (Krebs and Johnson, 1937; Cox and Clarke, 2014). When the acetyl group is oxidised to CO_2, the electrons released from this oxidation are taken up by the hydrogen (H^+) electron carrier NADH, which are generated as a result of the

acetyl group being oxidised to CO_2 (Alberts *et al.,* 2002). Subsequently, these high energy electrons from NADH are transferred to O_2, which reaches the cells from the lungs via the bloodstream. The O_2 uses the electrons, which then results in the O_2 being reduced to water (H_2O) in a series of reactions, which form the electron-transport chain (Tarnopolsky *et al.,* 2007; Cox and Clarke, 2014; Jeukendrup and Gleeson, 2018). The electron-transport chain takes place within the inner membrane of the mitochondria after the electrons and H^+ ions have been released from NADH and flavin adenine dinucleotide ($FADH_2$). The energy released by the transfer along the electron-transport train runs parallel with oxidative phosphorylation, which is the part of the electron-transport chain that synthesises most of a cell's ATP (Alberts *et al.,* 2002; Wilson, 2017; Mougios, 2020). When the electrons transport through the carrier proteins within the chain, these carrier proteins have the capacity to transport the majority of the H^+ ions within the inner membrane of the mitochondria, to the outer membrane (Alberts *et al.,* 2002). As a result of the movement of the H^+ ions, a concentration gradient is created, consequently, the H^+ ions transport back into the inner membrane of the mitochondria via diffusion through the ATP synthase (Mougios, 2020). Then, using the energy from the diffusion of the H^+ ions, ATP is produced. The ATP produced by the cells fuels the synthesis of ATP from adenosine diphosphate (ADP) and inorganic phosphate (P_i) via oxidative phosphorylation (Wilson, 2017; Mougios, 2020). In summary, due to carbohydrates, fats and proteins being burned by O_2 to CO_2 to produce energy, they are referred to as fuels for human cells, and therefore support human functioning, including exercise.

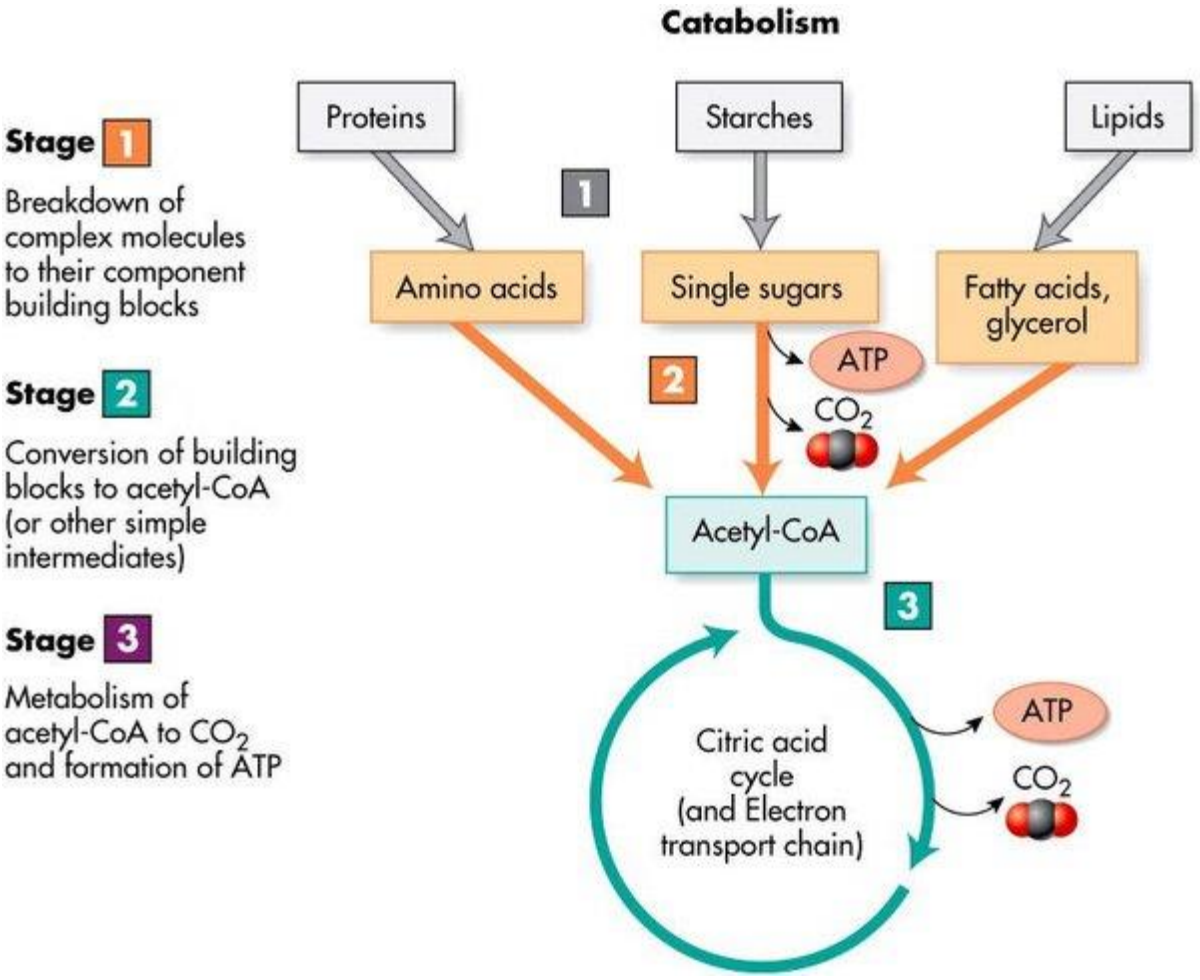

Figure 1: Schematic depiction of catabolism (adapted from Mougios, 2020). Figure 1 highlights the three key stages of catabolism that take place to produce ATP.

2.2.2 Production of ATP when O_2 is limited

When there is not sufficient volumes of O_2 to fuel the electron-transport chain/oxidative phosphorylation, a number of different processes take place to produce sufficient ATP. When there is not sufficient O_2, the process reverts back to when pyruvate is formed (Mougios, 2020). The pyruvate is then converted into lactate, which then buffers out the H^+ ions (Wilson, 2017). The buffering of H^+ ions prevents the blood from going acidic, which subsequently supports the uptake of metabolites such as O_2 and glucose to fuel exercise (Alberts *et al.*, 2002). Also, during the process of pyruvate being converted to lactate, NAD^+ is produced, which is then

reverted back to NADH when there is sufficient O_2 to support the process of synthesising ATP via the electron-transport chain (Wilson, 2017).

When humans have depleted glucose stores, several different processes (including anabolism and catabolism) take place to produce ATP. The depletion of glucose could be because an individual has used their stores (e.g., via exercise with no replenishment) or through consciously reducing carbohydrate intake (Mougios, 2020). Due to the limited glucose, no glycolysis can take place, which impacts the level of pyruvate produced, subsequently reducing the level of acetyl CoA (Alberts *et al.,* 2002). Consequently, the oxaloacetate transports out of the mitochondria (due to having nothing else to bind with) to produce more pyruvate to support glycolysis to replenish the lost glucose (Wilson, 2017). This then leads to the pyruvate trying to fuel glycolysis, with oxaloacetate concentrations reducing. Thus, the increased levels of concentrations of pyruvate increases the concentration of acetyl CoA, however it has no oxaloacetate to bind to. As a result of this and due to having depleted glucose levels, acetyl CoA levels accumulate and bind together and produce ketones, a process known as ketogenesis (Mougios, 2020). The ketones then transport out of the liver and move towards the brain. When the ketones transport through the blood-brain barrier, the ketones split apart (known as 'ketolysis') and then the ketones revert back to acetyl CoA and the Krebs cycle can then undergo its process (Krebs and Johnson, 1937). In addition to the ketones supporting the production of glucose, amino acids, glycerol and FAs also help. Specifically, amino acids are involved in aspects of glycolysis and the Krebs cycle, glycerol is involved in the process of glycolysis and FAs are involved minimally within the Krebs cycle to support the production of acetyl CoA (Alberts *et al.,* 2002; Shi and Tu, 2015; Mougios, 2020). The process of using sources other than carbohydrate sources to generate glucose is known as gluconeogenesis,

which is an example of anabolism. In turn, the body utilises amino acids, glycerol and FAs to support the production of ATP when glucose stores are low.

2.3 Manipulation of energy currency in endurance sport

Through manipulating an endurance athlete's training and nutrition effectively, sports scientists can elicit changes to an endurance athlete's metabolic flexibility and efficiency to support their performance. Metabolic flexibility is when an athlete has adequate availability and capacity to integrate the use of the muscle's fuel stores to produce ATP according to the demands of the event (Burke, 2021). Additionally, metabolic efficiency is an athlete's effectiveness to preserve as much energy as possible at a given workload, which is directly dependent on their metabolic flexibility (Cox and Clarke, 2014; Burke, 2021). An athlete's ability to utilise their substrates as effectively as possible is dependent on their metabolic flexibility, suggesting possessing a strong metabolic flexibility is more important than a strong metabolic efficiency for endurance athletes. Thus, essentially an athlete's endurance capacity is largely dependent on their metabolic flexibility, further emphasising its importance. The majority of an endurance athlete's training will be programmed at an aerobic intensity, consequently, endurance athletes will elicit aerobic-specific physiological adaptations that will stimulate their metabolic flexibility to maximise their metabolic efficiency (Kiens *et al.,* 1993; Burke, 2015; Cox *et al.*, 2016). When athletes reduce their carbohydrate intake before exercising aerobically, research has reported that athletes subsequently experience adaptations, such as increased cell signalling, presence of enzymes and increased fat oxidation to increase the sustainability of energy (Burke, 2015). Specifically, research has posited a significant increase of 41% ($P < 0.005$) in free fatty acid (FFA) concentration and a significant decrease in plasma glucose turnover during endurance exercise ($P < 0.001$) (Martin *et al*, 1993). The increased reliance on FFAs as a fuel source and the subsequent proposed benefits for endurance

athletes can be highlighted by the function of the aerobic energy system within the work of Randle (1963; Alberts *et al.,* 2002). Due to the increase in FFAs from beta oxidation, the mitochondria, the "powerhouse" of the cell where ATP is produced is signalled and subsequently changes its fuel preference due to the FFAs suppressing the pyruvate dehydrogenase complex (Bartlett *et al.,* 2013; Hawley and Morton, 2014). The suppression of the pyruvate dehydrogenase complex is instigated by the increased ratio of coenzymes including acetyl CoA/CoA and $NADH/NAD^+$, which have the function of increasing ATP production from the mitochondria via oxidative phosphorylation (White and Schenk, 2012; Hawley and Morton, 2014; Wilson, 2017). Consequently, there is a perturbation of ATP production within the mitochondria due to the increased presence of enzymes, which is suggested to support the sustainability of energy production, creating proposed benefits for endurance athletes (see mitochondrial biogenesis section for more information). Furthermore, having a higher reliance on fat as a fuel source is more conducive for endurance athletes because there is an increase in ATP availability (> 100 units of ATP per fat molecule in comparison to the 38 units of ATP per glucose molecule) (Wilmore *et al.,* 2016). Through having an increase in ATP availability, via an increased reliance on fat as a fuel source, it is suggested to delay the onset of glycogen depletion (Costa *et al.,* 2019: Bailey and Hennessy, 2020; Burke, 2021). This sparing of glycogen through the delayed depletion, could be useful during the decisive moments of competition, such as when overtaking an opponent or pursuing a "sprint finish" (Sansone *et al.,* 2018; Burke, 2021). Consequently, being able to spare carbohydrate is suggested to be a beneficial adaptation for endurance performance. The development of an endurance athletes' metabolic flexibility and efficiency was further explained within the 'crossover' concept of Brooks and Mercier (1994) (see Figure 2), whereby training under specific conditions, athletes are suggested to have a higher reliance on fat as the primary fuel source as intensity of exercise increases. In turn, endurance athletes rely on a

combination of carbohydrate and fat to fuel their activity, with the specific proportion of each substrate being dependent on an individual's metabolic efficiency, whereby they can spare carbohydrate for key moments of competition.

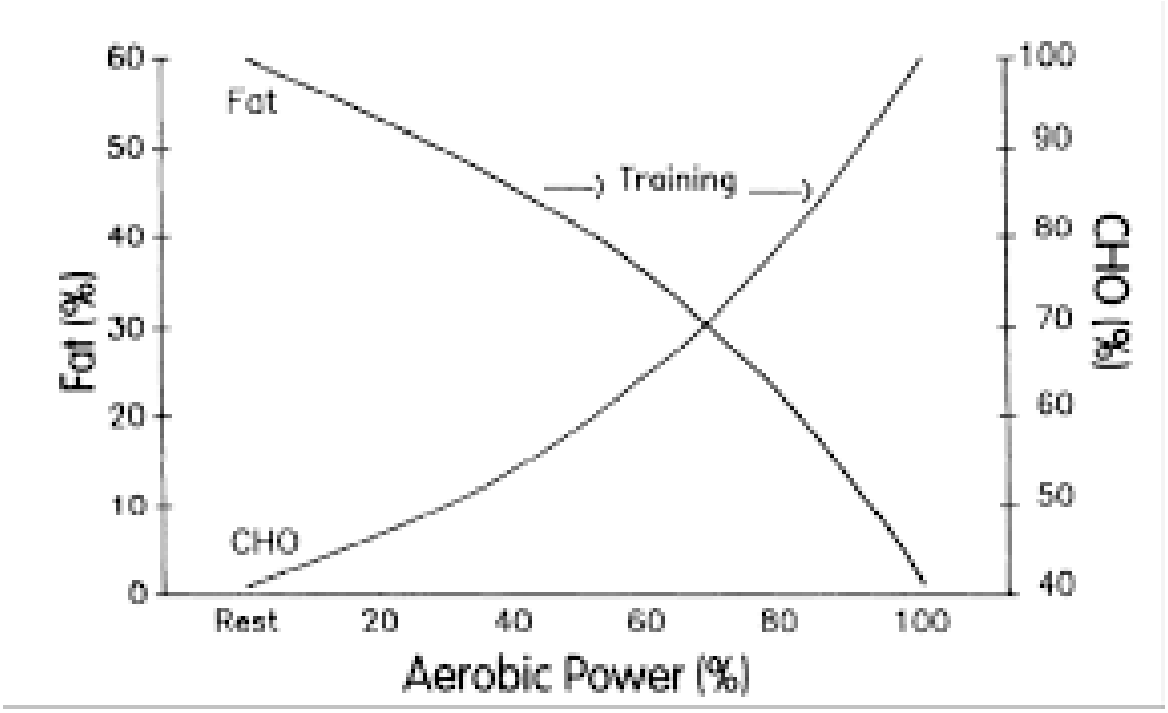

Figure 2: Brooks and Mercier's (1994) 'crossover' concept. On the x-axis is aerobic power (i.e., intensity of exercise), with the percentage of fat and CHO (carbohydrate) that is metabolised at a particular intensity. The 'training' arrows highlights that the point when fat metabolism exchanges into predominantly carbohydrate metabolism can occur at higher intensities when following specific training conditions.

2.4 Manipulating carbohydrate availability

Due to the research conclusively establishing that carbohydrate is a key factor for successful endurance performance it is important to understand how endurance athletes can maximise their carbohydrate availability. Furthermore, carbohydrate availability in the context of endurance exercise is well recognised, with the absolute concentration of carbohydrate to energy metabolism during exercise being dependent on the exercise intensity (Hawley and

Morton, 2014; Burke *et al.,* 2011). The work of Burke (2010; Burke *et al.,* 2011; Burke *et al.,* 2018) suggested the need to be pragmatic when deciding upon carbohydrate availability for training sessions and competitions. The need to be pragmatic was highlighted by the "train low compete high paradigm" (Hansen *et al.,* 2005), which proposes a continuum of carbohydrate intake relative to an individual's body mass, with the carbohydrate intake ranging from 1-12 $g \cdot kg^{-1}$ (Burke *et al.,* 2011). The continuum is split into two main parts, daily needs for fuel and recovery (training focus) and acute fuelling strategies (competition focus). The lower end of the continuum (1 – 7 $g \cdot kg^{-1}$) refers to fuelling for low-moderate-intensity training lasting ~1 h or immediate pre-competition fuelling (1-4 h; 1-4 $g \cdot kg^{-1}$) or immediate post competition refuelling (1 – 1.2 $g \cdot kg^{-1}$) (Burke *et al.,* 2011). As the workload increases in training programmes (i.e., 3-5 h of training at moderate-to-high intensity), a carbohydrate intake of 8-12 $g \cdot kg^{-1}$ is recommended, with an identical intake recommended for fuelling up 1-3 days before endurance events lasting ≥ 90 min take place (Burke *et al.,* 2011). In summary, the "train low compete high" paradigm informs practices today as to how to optimally fuel for training sessions and/or competition.

2.4.1 Competing high

From the train low compete high paradigm, it is suggested increasing carbohydrate intake immediately prior to an event supports endurance performance. Increasing carbohydrate intake, commonly referred to as 'carbohydrate loading' is where athletes consume 8 - 12$g \cdot kg^{-1}$ 3-5 days before an endurance event (Burke, 2010; Burke *et al.,* 2011). Following an acute 'carbohydrate loading' strategy has shown to increase glycogen content of an endurance athlete's muscle from 125$\cdot mmol \cdot kg^{-1}$ w.w. to values exceeding 220$\cdot mmol \cdot kg^{-1}$ w.w. (Rauch *et al.,* 1995; Hawley *et al.,* 1997; Bartlett *et al.,* 2015). Consequently, suggesting that an acute increase in carbohydrate intake results in a supercompensation of carbohydrate within the

muscle and liver. The supercompensation of muscle and liver glycogen is suggested to increase carbohydrate availability and improve endurance capacity by 20% and overall performance by 2-3% in endurance events lasting ≥ 90 minutes (Hawley *et al.,* 1997). Hawley and colleagues also suggested that the supercompensation of muscle and liver glycogen prior to competition is not required for events that last < 90 minutes. Thus, proposing glycogen supercompensation supports the majority of endurance events. The reasoning for the benefits of the supercompensation of muscle and liver glycogen for endurance events lasting > 90 minutes could be simply because of athletes possessing higher levels of carbohydrate to utilise for high-intensity activities over a longer period (Burke, 2021). Consequently, having spare resources of carbohydrate in comparison to if athletes did not super-compensate their liver and muscle glycogen stores. Also, the increased levels of carbohydrate could reduce the number of times to refuel during a race (Costa *et al.,* 2019; Bailey and Hennessy, 2020; Burke, 2020). Thus, saving time and potential gut disturbances due to the redistribution of nutrients (i.e., the blood from the working muscles to the splanchnic region), which is suggested to be caused by over fuelling during the race (Costa *et al.,* 2019; Bailey and Hennessy, 2020). Furthermore, concentrations of muscle glycogen are suggested to be a strong determinant for exercise performance highlighted by a strong correlation of r = 0.92 between muscle glycogen content and work time (Bergström *et al.,* 1967; Cox and Clarke, 2014; Burke *et al.,* 2017). Consequently, suggesting that 85% of an endurance athlete's work time is directly dependent on their muscle glycogen availability (r^2 = 0.85) further highlighting the importance to spare muscle glycogen for endurance performance. Overall, the literature suggests that carbohydrate loading supports endurance performance, which could explain why carbohydrate loading is a consistent intervention implemented by sports nutritionists for endurance athletes prior to competition (Hawley and Morton 2014; Bartlett *et al.,* 2015; Burke *et al.,* 2017).

2.4.2 Training high

In addition to the benefits of high carbohydrate availability for endurance competition, high carbohydrate availability has also been recommended before, during and after training sessions that are of high intensity and volume. High-intensity interval training is a pertinent training intervention for endurance athletes; thus, it is important to consider how endurance athletes fuel adequately for such sessions (Cox *et al.,* 2010; Mata *et al.,* 2019). The reasoning for a high intake of carbohydrate during periods of training programmes that are of high-intensity and volume is to optimise recovery and training-induced adaptations (Cermak and van Loon, 2013; Mata *et al.,* 2019; Burke, 2020). Furthermore, the study of Cox et al. (2010), which involved 16 male endurance-trained cyclists and triathletes compared the effects of a high carbohydrate diet vs a low carbohydrate diet over a 28-day period on exogenous carbohydrate oxidation during exercise. The findings reported the participants who followed a high carbohydrate diet had a significant increase in whole body glucose oxidation, with values of 54.6 to 63.6 g ($P < 0.01$), which could suggest why the high carbohydrate group significantly decreased their time trial time by 6% ($P < 0.01$) compared to their time trial time before following the 28-day diet. Contrarily to the HIGH carbohydrate group, in the LOW carbohydrate group there was with no difference found in the rate of exogenous glucose, however there was no difference in time trial time between groups. This finding could be because the time trial typically lasted between 25-30 minutes, which supports the rationale that the supercompensation of muscle and liver glycogen only effects performance of events lasting > 90 min (Burke, 2021). Despite these findings of Cox and colleagues supporting the propositions of other research, a limitation of this study was that the sample only included males, which lacks generalisability for the endurance-athlete population (Atkinson, 2012). Notwithstanding that there were no differences in performance outcomes between the groups in Cox et al.'s study, the research is unanimous for advocating a high carbohydrate diet when

the training programme is at a high volume and intensity for a sustained period. Moreover, if the recommended high intake of carbohydrate (6 – 12 g·kg^{-1}) is not followed during a sustained period of training of high-intensity and volume, athletes are at risk of suppressing their immune system, training adaptations and may ultimately decrease endurance performance (Baar and McGee, 2008; Burke *et al.,* 2011; Mata *et al.,* 2019). Thus, having high carbohydrate availability for competition and during high volumes of high-intensity training is posited to be strongly supportive for endurance performance. Conversely, if a high carbohydrate intake is not followed during periods of high-intensity training there are suggested to be damaging consequences to performance.

2.4.3 Training low

In addition to the perceived endurance performance and training benefits of high carbohydrate availability during high workloads, a recent growing body of research is suggesting consciously reducing carbohydrate availability (known as "training low") for an acute intermittently applied period (1-2 sessions at a time) enhances training adaptation and endurance performance. This concept was instigated by findings that highlighted conducive physiological adaptations, which supported athletes to have a greater sustainability of energy and enhance exercise capacity in endurance-trained individuals following an acute reduced carbohydrate intake accompanied by training sessions at reduced intensities < 80% $\dot{V}O_{2max}$ (Yeo *et al.,* 2008; Hulston *et al.,* 2010; Marquet *et al.,* 2016; Marquet *et al.,* 2016). The reported adaptations that stimulated the enhanced energy sustainability and exercise capacity included the enhanced activation of cell signalling pathways and increased levels of oxidative enzymes and fat oxidation. The idea of "training low" is grounded by the early work of Sherman et al. (1981) who found that endurance performance times were not affected with higher levels of muscle glycogen for a 20.9 km^{-1} run. Sherman and colleagues had six well-trained endurance

runners within their study, and the participants followed a nutrition strategy that consisted of either a 15% (low), 50% (medium) or 70% (high) carbohydrate intake before completing a 20.9 km^{-1} time trial. The trials were arranged into three (trial A, B and C) and were randomly assigned. Trial A was 3 days (low) vs 3 days (high); trial B was 3 days (medium) vs 3 days (high); trial C was 6 days (medium). Due to there being no difference in performance times during the 20.9 km^{-1} run between all trials, the results suggest that anything above the minimum required level of muscle glycogen for exercising at a given intensity and duration is unnecessary. Despite the limited generalisability of this study due to the small sample size (n = 6) and this finding only being relevant to running events that last specifically 20.9 km^{-1}, the findings of Sherman et al. created the foundation for the recent 'glycogen threshold' hypothesis of Impey and colleagues (2018).

The 'glycogen threshold' presents the idea of 'fuelling for the work required'. Fuelling for the work required reflects the idea within Sherman et al.'s (1981) study that suggested over fuelling in carbohydrate for what is required within the exercise that an endurance athlete is about to partake in is unnecessary. Impey and colleagues continued the earlier work of scholars such as Burke (*et al.,* 2011; Bartlett *et al.,* 2013; Hawley and Morton, 2014; Bartlett *et al.,* 2015) and translated the findings around carbohydrate intake and availability based around performance (Sherman *et al.,* 1981) and transferred them to training and maximising conducive adaptations through applying the 'glycogen threshold' to carbohydrate intake.

From this, 'fuelling for the work required' for training, whereby a level of muscle glycogen concentration of which training could be commenced within (i.e., the 'glycogen threshold'), could provide a metabolic milieu that maximises adaptations for endurance athletes (Burke *et al.,* 2011; Bartlett *et al.,* 2013; Hawley and Morton, 2014; Bartlett *et al.,* 2015; Impey *et al.,*

2018). When endurance athletes commence exercise with muscle glycogen that is too high for the work required, athletes have considerably more work to complete to elicit comparable cell signalling and muscle remodelling responses to those who commenced exercise with lower muscle glycogen (Bartlett *et al.,* 2015; Impey *et al.,* 2018). In short, when there is a discrepancy between the workload and intensity of the exercise and carbohydrate intake, endurance athletes are not maximising the adaptations of their training. Also, due to endurance athletes rarely completing identical sessions or training at maximum intensity during consecutive sessions, "training low" and 'fuelling for the work required' appears practical to implement and optimal for eliciting maximal adaptations during training programmes (Impey *et al.,* 2018). This can be ensured by manipulating carbohydrate intake acutely before key "train low" sessions so that endurance athletes can complete the sessions within the 'glycogen threshold' (Hawley and Morton, 2014; Bartlett *et al.,* 2015; Impey *et al.,* 2018). In summary, using the paradigms 'train low compete high' and 'fuelling for the work required', is suggested to be a pragmatic and practical approach for sports nutritionists and athletes to ensure optimum preparation and adaptations from their training.

Although there are suggested positives of "training low" for endurance adaptation, some research has reported "training low" to be ineffective and problematic for endurance athletes. Despite some literature suggesting favourable adaptations such as enhanced cell signalling, presence of enzymes, substrate use and exercise capacity, other research has reported such adaptations did not always occur (Burke *et al.,* 2015) and when adaptations did occur, they did not always translate to improved endurance-exercise performance (Wojtaszewski *et al.,* 2003; Yeo *et al.,* 2010; Psilander *et al.,* 2013; Jensen *et al.,* 2015; Lane *et al.,* 2015; Gejl *et al.,* 2017). However, some research has reported that adaptations did result in enhanced endurance performance, with increases in aerobic power and reductions in time trial times (Marquet *et*

al., 2016; Marquet *et al.,* 2016). Thus, the literature surrounding endurance exercise and acute reductions in carbohydrate intake is equivocal, with limited research focusing on the elite population. Moreover, "training low" has been shown to be problematic. For instance, "training low" has been shown to significantly reduce cognitive and immune function and increase muscle degradation ($P < 0.05$) if the "train low" strategy is followed long-term and if the absolute intensity of the training session whilst "training low" is too high (i.e., > 80% $\dot{V}O_{2max}$) (Costa *et al.,* 2005; Impey *et al.,* 2016). Subsequently, such ramifications could negatively affect future performance. The hypothesised debilitations in cognitive and physical function associated with "training low" could be due to confusion and lack of experience following such strategies within the athlete population (Impey *et al.,* 2018). Consequently, Burke et al. (2018) suggests the need for the athlete to gain education and experience using such practices and if possible have the assistance of sports nutritionists and sports scientists with "training low" knowledge and experience to support the implementation of the "training low" strategy. Having sports science led provision would ensure a critical absolute level of glycogen depletion is induced for significant activation of cell signalling pathways, whilst being able to exercise at the desired training workload and intensity without any maladaptation for athletes when "training low" (Burke *et al.,* 2018; Impey *et al.,* 2018). In short, sports science support would ensure endurance athletes elicit the optimum adaptations from "training low". "Training low" is suggested to create challenges for athletes, which could have negative impacts to the quality of adaptations they obtain. However, overall, "training low" within a hybrid approach for nutrition strategies ("compete high train low") is suggested to be a positive intervention for endurance performance, providing it is followed correctly with calculated and forward-planning implementation.

2.5 Mitochondrial biogenesis

Mitochondrial biogenesis is suggested to be the key adaptation following a period of "training low". Mitochondrial biogenesis refers to the increase in mass and number of mitochondria within endurance athletes' cells, and subsequently supports endurance athletes to exercise at higher absolute intensities for longer periods (Hawley and Morton, 2014; Bartlett *et al.,* 2015; Impey *et al.,* 2018). Specifically, the increased mitochondrial content within skeletal muscle reduces the level of disturbance to metabolic homeostasis at any given intensity, subsequently resulting in smaller decreases in ATP, phosphocreatine and carbohydrate utilisation, whilst increasing ADP, adenosine monophosphate (AMP) and lactate concentrations (Holloszy, 1967; Bartlett *et al.,* 2015). Thus, these adaptations support an endurance athlete's metabolic efficiency due to being able to maintain metabolic homeostasis more effectively, providing a greater consistent source of ATP for muscular contractions to be performed optimally for sustained periods.

2.5.1 Mitochondrial biogenesis during recovery

The transient changes in gene expression that occur during recovery after each training session of reduced muscle glycogen stimulate the increase in mitochondrial biogenesis, which may support endurance exercise performance (see Figure 3 for schematic). Firstly, the AMP-activated protein kinase (AMPK) and p38 mitogen-activated protein kinase (p38MAPK) enzymes, which ensure metabolic homeostasis by activating glucose and FA uptake and oxidation when cellular energy is reduced, phosphorylate at the nucleus and mitochondria (Bartlett *et al.,* 2015; Impey *et al.,* 2018). The phosphorylation of AMPK and p38MAPK subsequently stimulate increased expression of the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) enzyme, which supports nuclear-

encoded mitochondrial genes, in turn modulating metabolic processes through activating and translocating the mitochondria and nucleus (Holloszy, 1967; Perry *et al.*, 2010; Bartlett *et al.*, 2015; Marquet *et al.,* 2016). Furthermore, the suggested modulation of metabolic processes was supported by Safdar et al. (2011) who linked these adaptations to endurance exercise in mice. The study randomly assigned 12 mice who were matched for body mass into either a sedentary (SED) group, a forced-endurance exercise group (END) or a forced-endurance exercise group followed by 3 h of recovery (END+3h). Both the END and END+3 h groups exercised on a treadmill, running at 15 m·min for 90 min. After the exercise, ~200 mg of the quadriceps femoris muscle was extracted and analysed for mitochondrial and nuclear fractionation. In conclusion, the study reported a significant increase in activity and translocation of the nucleus and mitochondrial in the END and END+3 h groups in comparison to the SED group ($P < 0.05$). This finding suggests that endurance exercise stimulates PGC-1α, which strongly facilitates nuclear-mitochondrial DNA cross-talk, consequently supporting the turnover of mitochondrial biogenesis (Safdar *et al.,* 2011). From understanding that the increased expression of PGC-1α induces mitochondrial biogenesis within skeletal muscle, it could explain why studies have reported the overexpression of PGC-1α supports adaptations for endurance exercise. Reported adaptations for the overexpression of PGC-1α included the increase of fat oxidation, reduction in muscle glycogen utilisation during exercise and increase in $\dot{V}O_{2peak}$ and endurance capacity (Leone *et al.,* 2005; Wende *et al.,* 2007; Calvo *et al.,* 2008). Additionally, such findings could explain why deficiencies in PGC-1α reduce exercise capacity and increase fatigue (Leone *et al.,* 2005; Wende *et al.,* 2007; Calvo *et al.,* 2008). Overall, these findings suggest that AMPK and p38MAPK are potent regulators of mitochondrial content, function and biogenesis, which are suggested to support endurance exercise.

In addition to AMPK and p38MAPK being important regulators of mitochondria functioning, PGC-1α is recognised as a major point of control in regulating nuclear and mitochondrial DNA (mtDNA) expression. The PGC-1α collectively binds to and activates a number of transcription factors within the nucleus and mitochondria, which could assist metabolic efficiency when following a "train low" strategy (Bartlett *et al.,* 2015). Specifically, within the nucleus, PGC-1α stimulates transcription factors including nuclear respiratory factors 1 and 2 (NRF 1/2) to collectively induce the increase in proteins that are involved in the transport and oxidation of glucose and FAs (Impey *et al.,* 2018). Thus, when following a "train low" strategy, the adaptations from mitochondrial biogenesis (which are stimulated by reduced muscle glycogen concentrations) could support athletes to prioritise their use of FA stores rather than glucose due to the reduced carbohydrate intake and availability. This in turn, could support athletes to practice primarily metabolising fat during training and sparing muscle glycogen stores. Also, within the mitochondria, the PGC-1α controls the activity of mitochondrial transcription factor A (Tfam) to coordinate the regulation of the mtDNA and increase cytochrome c-oxidase (COX) subunit cellular content, which are important mitochondrial proteins of the electron transport train (Bartlett *et al.,* 2015; Impey *et al.,* 2018). Consequently, a gradient of H^+ ions can be generated. When a gradient of H^+ ions are generated a source of energy for reactions including the generation of ATP from the phosphorylation of ADP can take place, which would provide sustainable ATP production and therefore energy for athletes (Alberts *et al.,* 2002). These adaptations, which are predominately controlled by PGC-1α within the mitochondrial biogenesis process, are suggested to reduce disturbances to metabolic homeostasis and subsequently increase the sustainability of energy, which would support athletes during endurance exercise.

2.5.2 Mitochondrial biogenesis during exercise

As well as the mitochondrial biogenesis adaptations that take place during recovery after reduced carbohydrate availability, mitochondrial biogenesis has a potent regulator during exercise. The tumour suppressor p53 protein has recently been shown to be a potent regulator of mitochondrial biogenesis during exercise. This idea has been proposed by recent literature that has shown that the p53 protein modulates exercise performance, especially under conditions of low carbohydrate availability. The prominence of the p53 regulator under reduced carbohydrate availability was reported by Bartlett et al. (2013). Bartlett and colleagues reported due to the p53 regulator there was a significant increase in the expression of PGC-1α ($P < 0.05$) during exercise. The increase in the expression of PGC-1α during exercise is suggested to be a strong determinant of stimulating the perceived performance effects of mitochondrial biogenesis (Philp *et al.,* 2011; Safdar *et al.,* 2011; Philp *et al.,* 2013; Saleem and Hood, 2013). Bartlett et al. concluded that the exercise-induced increase in p53 phosphorylation could be due to the increased AMPK activity, with the increased AMPK activity being caused by the increased circulating FFA availability. Overall, these findings propose that the p53 protein strongly contributes to the regulation of mitochondrial biogenesis during exercise under reduced carbohydrate availability conditions.

Additionally, the role of adrenaline is suggested to play an important role in altering the mtDNA to manipulate substrate use during exercise in conditions of low carbohydrate availability that would be favourable for endurance athletes. Specifically, when exercising under conditions of reduced carbohydrate availability, there is an increase in circulating adrenaline concentration, which subsequently stimulates an increase in adipose tissue and intramuscular lipolysis (Bartlett *et al.*, 2015; Impey *et al.*, 2018). As a result of this elevation of FFA, the FFA activates the nuclear transcription factor, peroxisome proliferator activated

receptor delta (PPARD), which in turn increases the expression of proteins involved in lipid metabolism, including carnitine palmitoyltransferase1 (CPT-1), pyruvate dehydrogenase lipoamide kinase isozyme4 (PDK4), cluster of differentiation36 (CD36) and hormone-sensitive lipase (HSL) (Bartlett *et al.,* 2015; Impey *et al.,* 2018). Thus, instigating the increased use of fat during exercise. Moreover, when endurance athletes consume a pre-exercise or during exercise meal that is high in carbohydrate, these affects are reversed (Bartlett *et al.,* 2015). Consequently, as endurance athletes practice "training low", the alteration of mtDNA (i.e., increased expressions of enzymes that stimulate FFA use) are suggested to become more prominent to support athletes to use higher levels of fat during exercise, which could help endurance athletes to spare muscle glycogen for the most decisive moments of races.

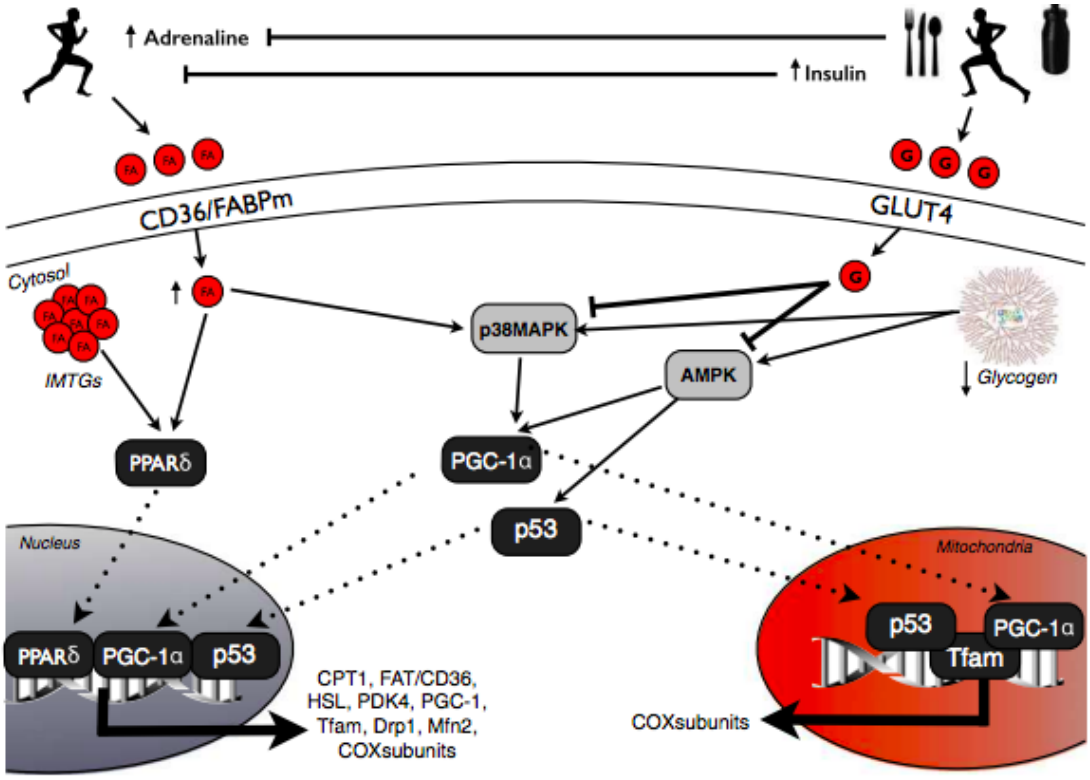

Figure 3: Schematic overview of mitochondrial biogenesis (cited from Bartlett *et al.*, 2015). The schematic shows the potential cell-signalling pathways with the roles regulating mitochondrial biogenesis when commencing endurance-based exercise under conditions of reduced carbohydrate availability.

2.6 Increase in ketone bodies via ketogenesis

In addition to mitochondrial biogenesis, the increased production of ketone bodies via ketogenesis during low carbohydrate availability instigates positive evolutionary effects that could be translated to enhancing endurance performance. This idea was grounded by the early work of Owen et al. (1967; Cahill 1970), who investigated the physiological adaptations within starving humans. The authors reported ketone molecules, acetoacetate (AcAc), betahydroxybutyrate and acetone are endogenously produced respiratory fuels and have the evolutionary effect to spare muscle glycogen stores, whilst themselves being a near endless

alternative energy source, thus profoundly increasing the capacity for survival (Cahill, 1968; Sansone *et al.,* 2018). The physiological underpinnings of increased ketones are stimulated when humans have low blood glucose and insulin levels, and consequently the liver responds by increasing the production of ketones (up to 150 - 185 $g \cdot d^{-1}$) (Cox and Clarke, 2014; Sansone *et al.,* 2018). This increase in ketone production results in the ketones acting as an alternative energy source. Cox and Clarke (2014) highlighted that such reliance on ketone bodies would be at a greater rate during fasted endurance exercise. In turn, these starvation physiology adaptations highlight how such findings could be translated to athletes who have low carbohydrate availability.

Despite the demands of starvation physiology occurring at an accelerated rate during ketogenic endurance exercise, the literature surrounding the application of ketone body metabolism within endurance exercise is limited. Both starvation physiology and ketone body metabolism depend upon glucose supply, with the concentrations of muscle glycogen being a strong determinant for endurance exercise performance, as previously established. When fuelling of each substrate is balanced (i.e., no deliberate restriction of any macronutrient), substrates compete to be metabolised by the working muscles. Carbohydrate is favoured as intensity increases, however as muscle glycogen content is decreased (e.g., after reducing carbohydrate intake), a concomitant increase (~20-fold) in blood ketones (i.e., betahydroxybutyrate) has been observed, with baseline levels typically being 0.1 mM, to 2-3 mM after both exhaustive and prolonged exercise, with the higher betahydroxybutyrate values being reported after sustained activity (Romijn *et al.,* 1995; van Loon *et al.,* 2001; Cox and Clarke, 2014; Cox *et al.,* 2016). It is important to note that these results were not following an acute reduced carbohydrate diet, but instead using exogenous ketones (Cox *et al.,* 2016). Although these findings do not directly relate to the topic of this present project, these foundational findings

do provide a suitable rational to suggest that if athletes were to follow a “train low” strategy, they may adopt the evolutionary adaptation to spare higher concentrations of muscle glycogen by using ketones and FAs as a primary and alternative substrate to provide energy to the working muscles.

Ketogenesis research within a sports setting is limited, however there is research to suggest being in a state of ketogenesis during exercise could benefit endurance athletes. Ketone bodies are metabolised by skeletal muscle and have a similar RQ to glucose (AcAc = 1.0 and betahudroxybutyrate = 0.89) if completely oxidised (Cox and Clarke, 2014). Also, ketone bodies do not require a glucose or FA transporter to enter the mitochondria, which is dissimilar to carbohydrate and fat (Frayn, 1983; Halestrap and Meredith, 2004; Cox and Clarke, 2014). Thus, when in a state of ketosis, ketone bodies have high accessibility to the mitochondria, which suggests ketone bodies could provide adequate energy for the working muscles when following a “train low” strategy. Most of the research with ketone concentration has been done with the regulation of ketogenesis and its role in starvation, obesity and diabetes (Cox and Clarke, 2014). Within the exercise setting, there are just a handful of reports for ketone concentration during whole-body exercise, from fewer than 30 people (Fery and Balasse, 1983; Fery and Balasse, 1986; Cox and Clarke, 2014). However, in a sporting context that shows relevance to this current project, Fery and Balasse (1983) analysed ketone body concentration involving overnight and 3-5 day-fasted untrained individuals before, during and after low intensity exercise (40 – 50% $\dot{V}O_{2max}$), which continued for either 30 min or 2 h respectively. The circulating betahydroxybutyrate levels dropped > 1 mM, with rates of disappearance and metabolism of ketones increasing five -to- eight-fold above resting conditions. Moreover, between 10.1 and 17.6% of CO_2 was derived from ketones within Fery and Balasse’s study, suggesting a significant oxidation of ketones in overnight fasted participants, even at low

workloads. The fasting and exercise protocol within the work of Fery and Balasse most closely reflects the protocols that will be explored in the current project. Moreover, the previous findings stimulate interest to investigate if endurance-trained athletes during a "train low" strategy would be able to minimise the level of ketone oxidation, thus preserving ketone stores for longer, and subsequently sparing muscle glycogen stores for greater sustained periods (Cox and Clarke, 2014; Sansone *et al.,* 2018; Bailey and Hennessy, 2020). The reported significant oxidation of ketones following a reduced carbohydrate intake, could support endurance athletes to adapt, utilise and conserve their energy more effectively to maximise training adaptations.

2.6.1 The ketogenic strategy

The ketogenic diet is a nutrition strategy within the nutrition literature and reflects the criteria for an acute "train low" strategy and obtaining biological ketosis. The ketogenic diet prescribes a significant reduction in carbohydrate intake, in turn facilitating physiological changes that promote the utilisation of ketone bodies and could elicit conducive physiological adaptations (Cox and Clarke, 2014; Bailey and Hennessy, 2020). In contrast to the peak value for relative carbohydrate intake being 12 $g \cdot kg \cdot d^{-1}$ within Burke et al.'s (2011) carbohydrate periodisation model, athletes on an acute ketogenic strategy will consume an absolute value ≤ 50 $g \cdot d^{-1}$ of carbohydrate. Additionally, to induce ketogenesis, a moderate protein intake of approximately 1.5 $g \cdot kg \cdot d^{-1}$ has been widely recommended (Sansone *et al.*, 2018). Currently, there is no relative or individualised value for carbohydrate "train low" recommendations, rather just an absolute approximate value of 50 $g \cdot d^{-1}$. Future research should aim to find more individualised "train low" recommendations. However, at present there are studies that have investigated the effect of the ketogenic diet on endurance adaptation and performance, however the use of an acute ketogenic strategy is rare within the literature. In the review of the ketogenic diet on endurance performance by Bailey and Hennessy (2020), the ketogenic diet was suited to induce

skeletal adaptations that have been previously highlighted within this literature review, with other findings suggesting that the ketogenic diet reduced lactate accumulation, thus supporting recovery and exercise capacity of athletes (Carr *et al.,* 2018; Ma *et al.,* 2018). Additionally, Phinney (2004) reported the ketogenic diet preserved time to exhaustion at 65% of $\dot{V}O_{2max}$ in endurance-trained individuals, whilst the RQ was 0.72, in turn suggesting that predominantly fat was being metabolised, thus instigating oxidative adaptations. Furthermore, González-Haro (*et al.,* 2007; Sansone *et al.,* 2018; Burke, 2021) reported following suitable training and nutrition interventions, fat oxidation rates have shown to increase from 0.4-0.6 $g \cdot min^{-1}$ at 40-60% $\dot{V}O_{2max}$ to 1.5 $g \cdot min^{-1}$ at 70% $\dot{V}O_{2max}$. Consequently, highlighting an increase in the reliance on fat at higher intensities. Despite these findings being from a chronic ketogenic diet (6 – 8 weeks), the results suggest that an acute ketogenic strategy could supplement the stimulation of conducive physiological benefits for endurance athletes whilst "training low". Furthermore, due to the "train-low" strategy being a short-term manipulation of carbohydrate intake, the "training low" strategies, which can include low or limited carbohydrate intake, should not be confused with a low-carbohydrate-high-fat-diet (LCHF). The LCHF diet is a longer-term, habitual diet that has shown similar benefits to "train low" strategies (Burke *et al.,* 2018; Burke, 2021). Overall, the ketogenic strategy is suggested to be suitable for endurance exercise when the intensity of the exercise is at a low-to-moderate intensity (< 80% $\dot{V}O_{2max}$) (Wilmore *et al.,* 2016), to support the optimum suggested physiological benefits of "training low".

2.7 Assessing low carbohydrate availability

There are various methods by which carbohydrate availability is assessed in a sports and exercise science setting. The most common methods for assessing the level of substrate use at

rest or during exercise include muscle biopsies, gas analysis (i.e., respiratory exchange ratio) and blood markers (e.g., glucose, triglycerides) which have been widely researched and noted as suitable methods (Goedecke *et al.,* 2000; Burke *et al.,* 2011). Recently, newly developed and innovative methods including BrAce detection and more commonly used methods in the sport and exercise setting including blood ketone markers are being used to analyse substrate use more frequently. In addition to gas analysis, this present study will be using BrAce and betahydroxybutyrate measurements to analyse the metabolism of participants to investigate their practicality and proficiency within a sport and exercise science setting.

2.7.1 Breath acetone detection

Due to the scientific grounding previously discussed around ketonic metabolism and the use of BrAce detection with the treatment and management of diseases related to cellular glucose, using BrAce detection could be a new, innovative way to assess carbohydrate availability in athletes. This study is developing a protocol to investigate whether a real time BrAce detector can effectively detect low carbohydrate availability. This in turn, would support athletes to commence exercise in a "train low" state, to ensure they are maximising the suggested physiological adaptations. Currently, effective BrAce detectors are lacking, with detectors (such as the prototype involved in this study) still being in the early development phases (Alkedeh and Priefer, 2021). Thus, suggesting the importance to investigate BrAce levels linked to training low to support the development of BrAce practices moving froward. Acetone is created as a result of a chain of chemical processes. After the hepatic oxidation of FAs there is an accumulation of acetyl CoA, which then catabolises to AcAc (Evans *et al.,* 2017). Subsequently, the AcAc undergoes decarboxylation and enzymatic degradation to betahydroxybutyrate and acetone (Sansone *et al.,* 2018). Due to its small molecular size, acetone is transported to the lung and excreted as an exhaled gas

(Anderson, 2015). The use of BrAce has mainly been used for the detection, treatment and management of diseases such as diabetic ketoacidosis and obesity. Using the example of diabetic ketoacidosis, a condition that is related to the theme involved in this study of reduced carbohydrate availability, is where patients fail to produce enough insulin, which has the function of transporting glucose into the cells (Anderson, 2015; Alkedeh and Priefer, 2021). Consequently, similar to the ideas presented by Owen et al. (1967; Cahill and Owen, 1968; Cahill, 1970) the body responds to the cells receiving reduced concentrations of glucose by producing ketones. Moreover, normal values of acetone following a balanced diet is 0 – 2 ppm, whereas diabetic ketoacidosis patients can have acetone values of ~1250 ppm (Alkedeh and Priefer, 2021). This highlights the range in which acetone values are produced following the use of BrAce. The level of BrAce produced is individual dependent, further highlighted by the range of BrAce levels reported within the literature reviews of Anderson (2015) and Alkedeh and Priefer (2021) when individuals were following a ketogenic strategy. The authors reported differences in calorie restriction within the ketogenic strategy, with basal metabolic rates and training levels being the key factors for the differing levels in BrAce concentrations. Specifically, the greater restriction in calorie intake whilst following the ketogenic strategy and the more highly trained an individual was, the higher their BrAce concentrations were. Furthermore, the strong correlation of BrAce with plasma AcAc and betahydroxybutyrate ($r = 0.77$, $P < 0.0001$) suggests that BrAce is a strong predictor of ketosis (Musa-Veloso *et al.*, 2002; Španěl *et al.*, 2011; Anderson, 2015). Thus, BrAce detection provides a suitable rationale to monitor ketone production, that could ensure athletes are in a ketogenic state when exercising to elicit the perceived physiological adaptations from “training low”.

Despite the effectiveness of BrAce with illnesses and conditions, BrAce detection within a sport and exercise setting for assessing carbohydrate availability is limited. Within the sport and exercise setting, the current 'gold-standard' method for assessing carbohydrate availability is a muscle biopsy to measure muscle glycogen content (Burke *et al.,* 2011; Jeukendrup and Gleeson, 2018). However, taking a muscle biopsy is invasive and the analysis of muscle glycogen content from a biopsy takes considerable time (Burke *et al.,* 2011). In contrast to the muscle biopsy, BrAce detection provides an immediate and real-time measure of BrAce, highlighting carbohydrate availability (Königstein *et al.,* 2020). Furthermore, BrAce detection is less invasive, due to the participant simply having to breathe into a mouthpiece (Königstein *et al.,* 2020). Consequently, BrAce seems promising for real-time monitoring of metabolic processes before, during and after exercise. Particularly, acetone is closely related to fat metabolism, highlighted by the strong correlation of $r = 0.88$ ($P < 0.001$) between acetone and fat oxidation during prolonged exercise (Sasaki *et al.,* 2011). In turn, further highlighting the usefulness of BrAce for endurance exercise.

In addition to the findings that suggest that BrAce detection has been effective within a sport and exercise science setting, BrAce detection has also shown to effectively distinguish between varying levels of fat metabolism between trained and untrained participants and when following low vs balanced carbohydrate diets. Königstein (*et al.,* 2020) posited that individuals who possessed higher aerobic fitness had higher increases in BrAce than that of BrAce concentrations in individuals who possessed lower aerobic fitness before, during and after exercise. In high aerobic-trained participants, their BrAce increased by $33\% \cdot h^{-1}$ vs $0.2\% \cdot h^{-1}$ in low aerobic-trained participants. In turn, these findings suggest that higher-aerobic-trained individuals had stronger exercise-related activation of fat metabolism, which corresponds to the literature and further highlights that BrAce could be a valid method to assess the level of carbohydrate availability in endurance athletes. Notwithstanding the

perceived validity of using BrAce from Königstein and colleagues' study, the participants did not follow a low carbohydrate diet. However, Ota et al. (2020) performed a study that compared BrAce levels between non-endurance participants who followed either a reduced carbohydrate diet or a balanced diet before performing a sprint protocol. Ota et al. reported a significantly higher BrAce after sprinting in the participants who followed a reduced carbohydrate diet in comparison to that of the participants that followed a balanced diet ($P < 0.05$). Thus, these findings suggest BrAce could be effective in detecting low carbohydrate availability in endurance athletes to support them in eliciting favourable physiological adaptations for endurance performance. Overall, BrAce provides an alternative and non-invasive procedure to evaluate changes in fat metabolism and therefore carbohydrate availability pre-, during and post-exercise.

2.7.2 Betahydroxybutyrate

Assessing betahydroxybutyrate concentration is another measurement that can be taken to analyse carbohydrate availability and the level of ketosis in athletes. This method is more invasive than BrAce, due to the process requiring a capillary blood sample, which will then be analysed by a ketone meter, which immediately analyses the betahydroxybutyrate content via electrochemical detection (Moore *et al.,* 2021). Notwithstanding that this method of acquiring betahydroxybutyrate is invasive, such a method has shown to be optimally valid for individuals with betahydroxybutyrate levels of 0-3 mM, which is a common measurement found in individuals following an acute ketogenic diet (Veech, 2004; Janssen *et al.,* 2010; Moore *et al.,* 2021). Thus, this method of obtaining betahydroxybutyrate is suggested to work consistently with an acute "train low" strategy, consequently making it a suitable method to

use in conjunction with BrAce detection to analyse the level of ketosis within endurance athletes.

CHAPTER 3 – DEVELOPMENT OF MOUTHPIECE AND INTEGRATION OF CHEMISTRY EQUIPMENT WITH SPORTS SCIENCE EQUIPMENT

3.1 Introduction

Due to the range of measures that were required from both chemistry and sports science equipment, it was essential in the development of the overarching protocol to understand how to integrate the equipment in the most effective way. The CELIF BrAce detector, Ketonix® and Cortex had their own mouthpieces that were only compatible for their own machine (see Figures 4 to 6). Specifically, the research team wanted to understand if it was possible to collect the CELIF BrAce detector and Cortex measurements in parallel. In addition to saving time by taking these measurements in parallel, the removal and re-attachment of the facemask (Hans Rudolph V2, USA, see Figure 7 and Figure 8) was proving to be an inconvenience, simply due to the amount of time it was taking to remove and reattach the mask. It was understood that the Ketonix® measurements were required to be taken separately as it would not be practical or possible to take the three measurements at once. Additionally, it was understood that blood measurements could be taken during the time that the participant was providing the $\dot{V}O_2$, $\dot{V}CO_2$ and BrAce measurements. Furthermore, the aim of Chapter 3 was to establish the most efficient procedure for collecting the required gas samples.

Figure 4: The mouthpiece that is compatible with the CELIF BrAce detector.

Figure 5: The Ketonix® with the accommodating mouthpiece attached.

Figure 6: A pilot participant demonstrating how the mouthpiece connects the gas inlet line from the CELIF BrAce detector to the participant's breath.

Figure 7: Hans Rudolph V2 mask (left), compatible with the Cortex M3B.The turbine (right) that collects the air for analysis by the M3B connects into the hole within the Hans Rudolph V2 mask.

Figure 8: A pilot participant demonstrating how the turbine from the Cortex M3B connects to the Hans Rudolph V2 facemasks to collect gas analysis.

3.2 Methods

To try and address the idea of taking the CELIF BrAce and Cortex measurements simultaneously, a LemonMedical adaptor (see Figure 9) was used to connect the Cortex turbine and the gas inlet line from the CELIF BrAce detector. Firstly, a hole was drilled within the LemonMedical adaptor, which was the perfect diameter for the gas inlet line to fit in the LemonMedical adaptor, to prevent any air leaving the hole without going into the gas

inlet line. Masking tape was used and was forcefully placed in the cortex turbine into the LemonMedical adaptor to successfully connect the CELIF BrAce detector and Cortex together (see Figure 10). Finally, the research team tried to take measurements at rest and during exercise to see if the data could be collected in parallel (Table 1), and this data was compared to data, which used the CELIF BrAce gas inlet and accommodating mouthpiece independently from the Cortex (Table 2).

Figure 9: LemonMedical adaptor, which has been adapted through drilling a hole that is identical to the diameter of the gas inlet line from the CELIF BrAce detector.

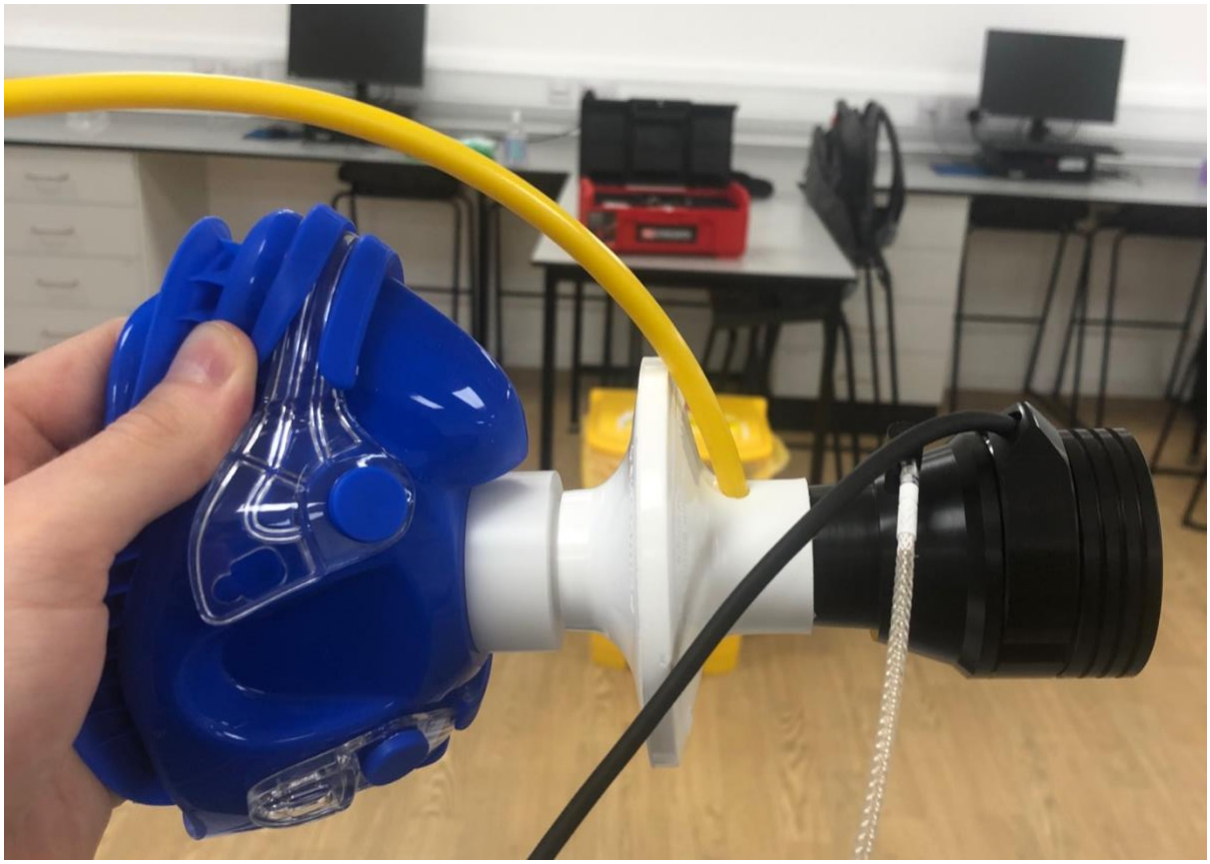

Figure 10: The LemonMedical adaptor attached to the gas inlet line of the CELIF BrAce prototype and the turbine and accommodating Hans Rudolph V2 facemask. This allows the CELIF BrAce acetone concentrations and cortex gas analysis to take place in parallel.

3.2.1 BrAce detection

The first BrAce detector that was used in this study, was the CELIF BrAce prototype, which was developed by the Department of Chemistry at Durham University (see Figure 11). The CELIF technique to ascertain acetone integrates two techniques, which are further explained below.

3.2.2 Cavity Enhanced Laser Induced Fluorescence

The CELIF is a technique that was used in this study to ascertain the level of acetone concentration within the participant's breath. The CELIF technique involves a combination of Cavity Ring Down Spectroscopy (CRDS) with Laser Induced Fluorescence (LIF) to determine the absolute acetone concentration (Mizouri *et al.,* 2013). Firstly, CRDS uses direct absorption, which allows the determination of the absolute acetone concentration (Sanders *et al.,* 2018). The CRDS functions by a laser beam being guided into an optical cavity, which has highly reflective mirrors (≥ 0.999 reflectivity) (Mozetič *et al.,* 2019). Then, the laser turns off, and the light intensity in the cavity exponentially decays as it escapes through both cavity mirrors. The exponential decay (known as a ring-down) is detected by a photodetector and the time constant of the decay (the ring-down time) measured (PICARRO, 2022). If a light-absorbing material (i.e., acetone) is placed into the cavity, the ring-down time decreases in comparison to a cavity with no additional gases due to fewer bounces of light being required before the light is fully lost from the cavity (Sanders *et al.,* 2018; PICARRO, 2022). The concentration of acetone can be determined from the difference between the two ring-down times. However, there is uncertainty in the determination of ring-down times caused by experimental noise. If the difference between the two ring-down times is consistent with zero, within experimental uncertainty, then the measurement is below the lower limit of sensitivity (Leeds University, 2022; PICARRO, 2022). The larger the uncertainty, the larger the absorbing molecule concentration has to be such that the two 'ring-down' times can be distinguished from each other, i.e., their difference is not consistent with zero (Leeds University, 2022; PICARRO, 2022). When absorption is very high at the upper limit of the dynamic range for CRDS, light comes into the cavity, and is completely absorbed such that it is not possible to measure a ring-down time (Carty, private communication).

The LIF is a highly sensitive technique, with a dynamic range much larger than the CRDS, which follows two steps; the absorption of a laser photon followed by the emission of a fluorescence photon from the excited state (Andresen and Strube, 1994). Absorption of a laser photon by the acetone produces an electronically excited state, which subsequently radiates. The fluorescence emission is detected using a filter or a monochromator followed by a photomultiplier (Crosley, 1982). The number of fluorescence photons emitted is proportional to the concentration of the emitting molecule. The lower limit of LIF is caused by a dark count background rate, whereby the photon counter cannot distinguish the fluorescence of acetone and other background sources of light (e.g., cosmic rays, scattered light from the laser or fluorescence of any materials illuminated by the laser). Although measures are taken to minimise the fluorescence disturbance from sources other than the acetone, it is difficult to prevent. Additionally, LIF has an upper-limit of sensitivity. Due to the time-frame in which photons are being counted during 'ring-down', a lot of photon counts can occur during this period before the photons start clashing into each other. When the photons clash into one another the counter cannot distinguish between multiple clashing photons. Such 'photon coincidences' can lead to the undercounting of photons and a loss of proportionality between concentration and photon count. Consequently, the upper-limit of LIF is when the molecule concentration is below the point where undercounting becomes significant (Carty, private communication).

CELIF, as applied to acetone, uses a 266 nm Nd:YAG laser in an optical cavity, which interacts with the sample for both the fluorescence and ring-down measurements (Sanders *et al.*, 2018). Consequently, this technique allows the combination of the absolute concentration measurement capabilities of CRDS with the sensitivity of LIF to measure the absolute

absorption concentrations (ppb or ppm) of acetone within a dynamic range of 500 ppb – >2000 ppm (Carty, private communication).

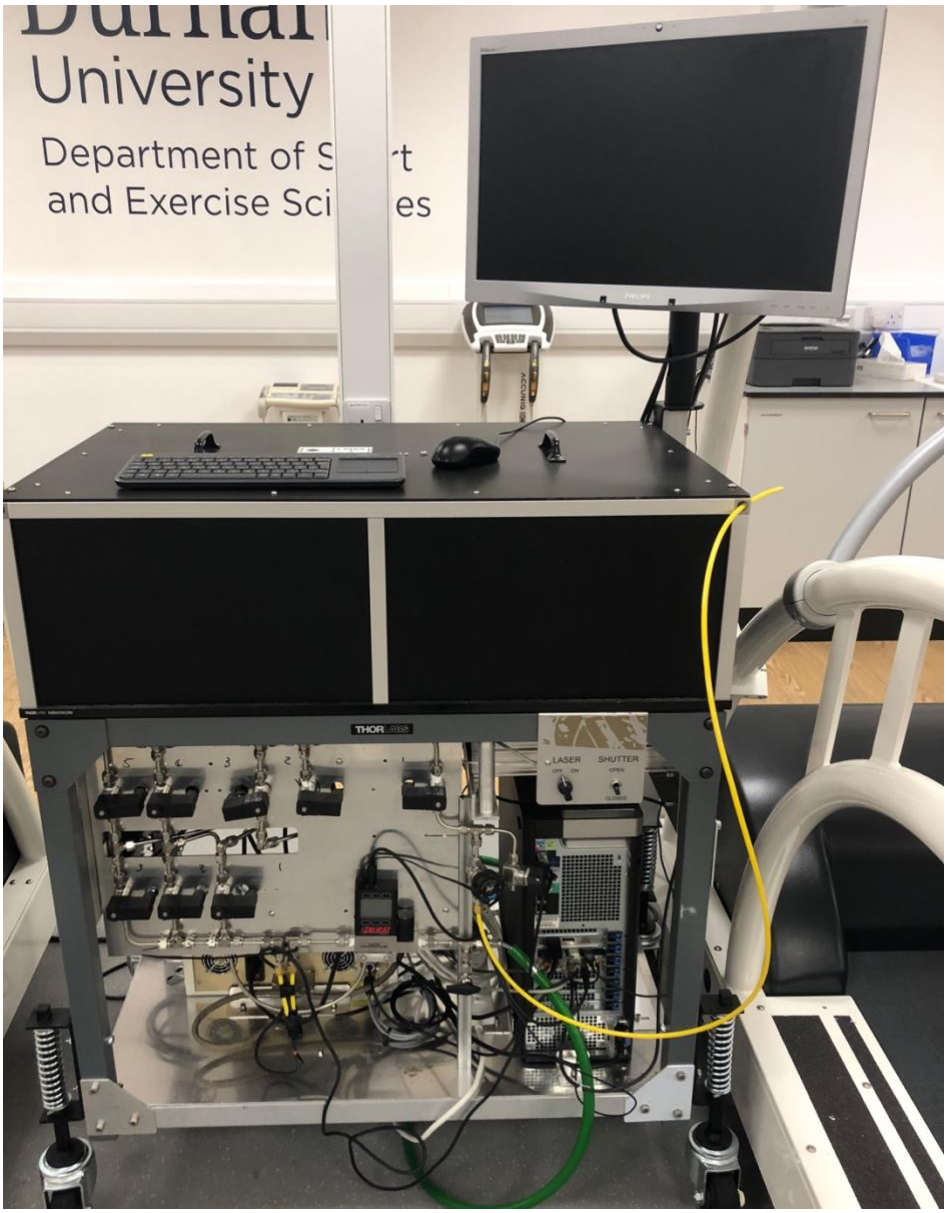

Figure 11: The CELIF BrAce prototype developed by the Department of Chemistry at Durham University.

3.2.3 Ketonix®

The Ketonix® (Varberg, Sweden) is a portable Bluetooth BrAce detector, which is currently in the market. This study is aiming to compare the measurements of the CELIF acetone

machine and the Ketonix®. The Food and Drug Administration (FDA) state the Ketonix® device is a registered Class 1 device, which means that the Ketonix® is low risk and requires general controls, highlighting the accessibility of the device to the general population (Qualio, 2022). The participant exhales into the device for as long and as hard as they can (i.e., an end-tidal breath, Ketonix®, 2022). As the participant is blowing into the device, a built-in LED then changes colour based on the BrAce level (green light = small trace, yellow = moderate trace, and red = high trace) (see Figure 12) (Peake *et al.*, 2018).

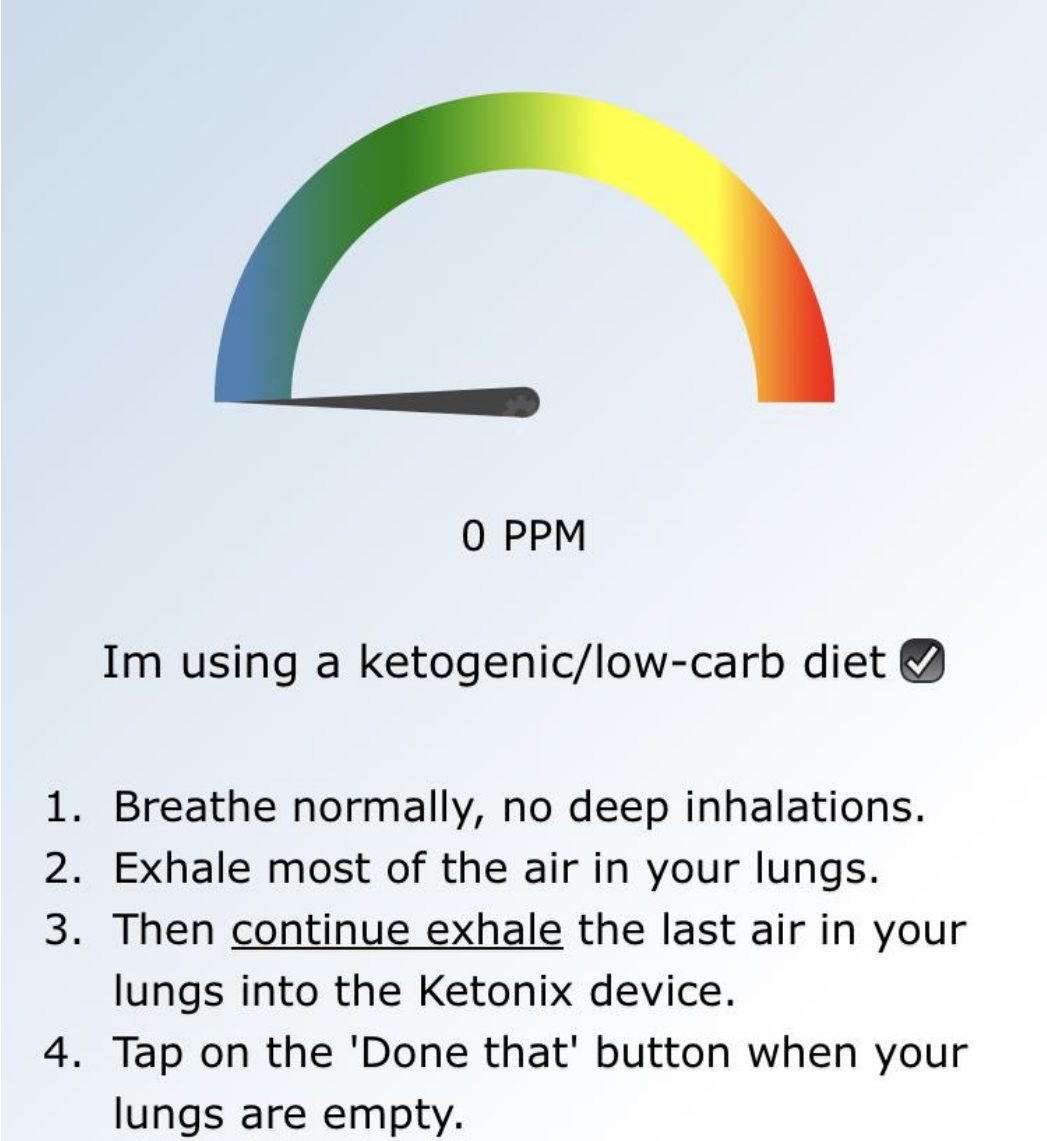

Figure 12: The interactive screen within the Ketonix® app that signifies the BrAce concentration level. Below the rainbow wheel are the instructions, which the lead researcher used to explain to the participants involved throughout this methodological-development study before they began their single end tidal exhalation.

3.2.4 Gas analysis – at rest and during exercise

In comparison to the primary criterion douglas bag method (DBM) for gas analysis, the Cortex Metalyzer 3B (Cortex, Germany) (M3B) has been shown to provide reliable and valid measurements for gas analysis at rest and for $\dot{V}O_{2max}$ tests, whilst also being the more practical method for this present study. The studies of Vogler (*et al.*, 2010) and Macfarlane and Wong (2012), which compared the DBM and M3B methods showed significant but small differences found for expired ventilation ($\dot{V}_E$), $\dot{V}O_2$ and $\dot{V}CO_2$ at all levels of frequencies. Furthermore, within Macfarlane and Wong's (2012) study, the differences between DBM and M3B were consistently < 2.5%, whilst the average intra-device TEM for $\dot{V}_E$, $\dot{V}O_2$ and $\dot{V}CO_2$ were 0.2, 1.4 and 1.1%, respectively. This highlights how although M3B method is not as valid as the DBM method, using the M3B is an effective alternative for gas analysis during a $\dot{V}O_{2max}$ test and for understanding substrate use during exercise and at rest. Furthermore, the M3B method is suggested to be more practical, due to the M3B presenting faster gas analysis than the DBM methods (Wilmore *et al.*, 2016). This was particularly useful for this present study whereby multiple gas analyses are required.

3.3 Results

Table 1 and Table 2 provide the BrAce results from using either the LemonMedical adaptor (Table 1) or the CELIF BrAce without the LemonMedical adaptor (Table 2).

Table 1: Results from the LemonMedical adaptor. Note the participant was not fasted.

File name	**No.Breaths**	**$ETCO_2$(%)**	**BrAce(ppm)**	**Error(ppm)**
Run01-pre-redone.txt	13	10.98	-25.57	0.78
Run01-post.txt	1	8.81	-0.55	0.93
Run01-post-2.txt	1	8.07	-0.08	1.07

Table 2: Results from the CELIF BrAce inlet line with compatible mouthpiece. Note the participant was not fasted.

File name:	**No.Breaths**	**$ETCO_2$(%)**	**BrAce(ppm)**	**Error(ppm)**
2022-05-31 Test-02.txt	37	10.75	0.58	0.3
2022-05-31 Test-03 mask.txt	47	12.17	0.31	0.34
2022-05-31 Test-04 mask.txt	54	7.34	0.18	0.23

Table 1 and 2 highlight the BrAce concentration results from the CELIF prototype when the LemonMedical adaptor was used (Table 1) and when the CELIF measurements were taken without the LemonMedical adaptor (Table 2). The results in Table 1 present data that was did not reflect the data in Table 2. The number of breaths and BrAce concentrations are different in comparison to what Table 2 presents, which questions the usefulness of the LemonMedical adaptor.

3.4 Discussion

Overall, the results suggested that the implementation of the LemonMedical adaptor affected BrAce values through, which is highlighted in the differences in BrAce values and number of breaths between the trials. Thus, moving forward the protocol during exercise involved measurements being taken sequentially.

3.4.1 The CELIF BrAce and Cortex

The number of breaths, end tidal CO_2 ($ETCO_2$(%)) and BrAce measurements were impacted due to the 'dead space' from the LemonMedical adaptor. During Run01-post and Run01-post-2 (see Table 1) acetone samples were taken for 120 seconds, and as is highlighted from Table 1, the number of breaths are not a true reflection of the number of breaths the participant actually completed. Conversely, Table 2 shows the expected results given the non-fasted state of the individual. Consequently, from analysing the data in Table 1 and comparing the data to Table 2, moving forward, the research team needed more viable equipment, so that the $ETCO_2$ is not impacted, as well as the number of breaths.

The data from Tables 1 and 2 suggests it would be suitable to trial other options of facemasks and to also take $\dot{V}O_2/\dot{V}CO_2$ from the cortex and CELIF BrAce sequentially rather than in parallel. To remove the inconvenience of the Hans Rudolph (V2, USA) facemask, moving forward, the study used a nose-clip (Hans Rudolph, 9014, USA) and a mouthpiece (Hans Rudolph, 9060, USA) that was compatible with the Cortex turbine to obtain gas analysis measurements (see Figure 13). Additionally, the CELIF BrAce mouthpiece will be used to acquire acetone measurements. The use of a Hans Rudolph nose-clip with the Hans Rudolph mouthpiece will accentuate the use of breathing from the mouth, which should support the

Cortex and CELIF BrAce measurements. Additionally, less ‘dead space’ is present when using this new protocol, and the ‘dead space’ that exists will be accommodated for within the set-up of the Cortex M3B. A negative for using this Hans Rudolph mouthpiece instead of the V2 facemask, is the increase in saliva that the participant will produce due to the accentuation of mouth breathing, which may impact the level of gas that is detected by the Cortex, which may affect the effectiveness of the measurements. In summary, implementing the Hans Rudolph mouthpiece that is compatible with the Cortex M3B turbine, will prevent the issues of ‘dead space’ that was presented when using the LemonMedical adaptor to try and take all measurements in parallel.

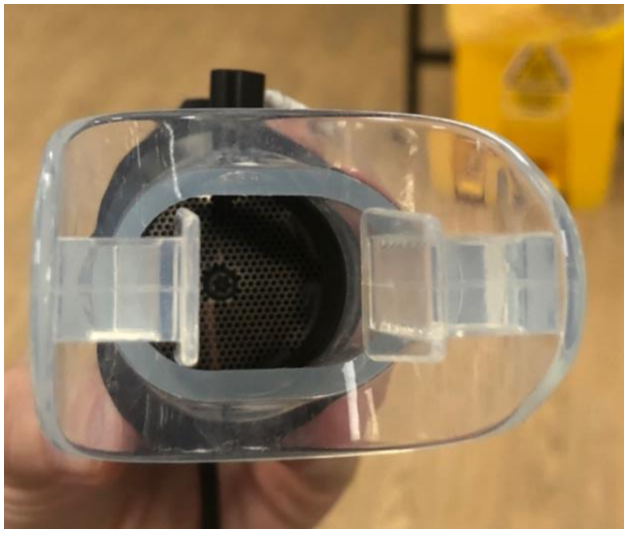

Figure 13: The Hans Rudolph (9060, USA) mouthpiece and nose clip that was used to connect to the turbine and M3B.

3.4.2 Ketonix®

As already stated, the Ketonix® measurement will be taken separately to the CELIF BrAce and Cortex measurements. To prevent the need to sterilise the mouthpiece of the Ketonix® after its every use, the CELIF BrAce mouthpiece was integrated with cardboard and tape to ensure the mouthpiece fitted properly and that the hole of the mouthpiece was covered to prevent air loss (see Figure 14). As a result, this made using the Ketonix® more practical for when multiple participants are assessed in short periods (i.e., untrained participant BrAce measurements as shown in Chapter 5).

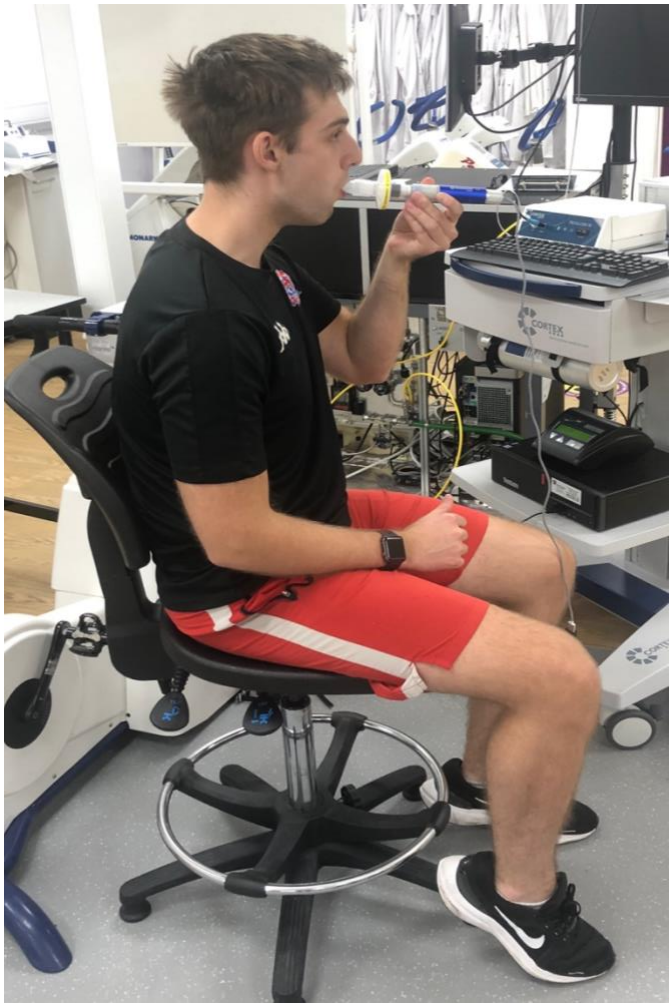

Figure 14: The Ketonix® with the CELIF BrAce mouthpiece. Left figure: The mouthpiece that was used for the CELIF BrAce can be used with the Ketonix®, via some cardboard and tape, to ensure maximum attachment and to minimise 'dead space'. Also, through placing the hole of the mouthpiece over the tape will minimise air lost from the participant's breath. Right figure: Pilot participant using the Ketonix® with the CELIF BrAce mouthpiece.

CHAPTER 4 – DEVELOPMENT OF PROTOCOL DURING EXERCISE

4.1 Introduction

From establishing that the gas samples required to be collected sequentially, the next task was to identify the most effective sequence to collect all the measurements. As already identified, this method-focused study involved a number of different measures, and to be as practical as possible, measurements needed to be taken in unison during exercise. Specifically, both blood and breath measurements needed to be taken and analysed simultaneously whilst the participant was exercising. This contrasts the protocol prior to exercising where all the measurements were planned to be taken sequentially. Moreover, before the exercise in the experimental protocol, resting capillary blood glucose, betahydroxybutyrate, BrAce (CELIF and Ketonix®) and $\dot{V}O_2/\dot{V}CO_2$ will be taken before they commence exercise. Due to the complexity of taking the measurements during exercise, it was important to know the most effective way to obtain the measures before official data collection for the experimental protocol began. As a research group it was discussed that if all the measurements took a prolonged time, it could impede a participant's ability to exercise, consequently negatively impacting the validity of the results. Due to the potential consequences of not collecting data effectively, it was decided to trial and understand the most practical order to take the different measurements, and how-to set-up the equipment (i.e., the positioning of equipment). As such, this chapter aimed to identify the most effective sequence to collect the measurements required.

4.2 Methods

To address the aim of understanding the most effective sequence to collect the measurements required, a practice protocol was trialled for two pilot participants whilst they were exercising.

4.2.1 Ordering of measurements

Trial 1 below highlights the protocol that was used whilst two participants were exercising at a moderate intensity (65% $\dot{V}O_{2max}$) for 30 min (Burke *et al.*, 2011). Measurements were taken at 10 and 20 min whilst the participants were exercising. The lead researcher who was taking the measurements was supervised by a trained lab technician to support the taking of measurements and provide suitable recommendations moving forward to support the effectiveness of acquiring the measurements.

4.2.2 Trial 1:

1. $\dot{V}O_2/\dot{V}CO_2$ with bloods (glucose, triglycerides and betahydroxybutyrate) (60 s worth of measurement)
2. CELIF BrAce (60 s worth of measurement)
3. Ketonix® (60 s worth of measurement)

The reasoning for this order was because the $\dot{V}O_2/\dot{V}CO_2$ Cortex M3B measurements required the least amount of preparation. All the lead researcher had to do was place the turbine into the Hans Rudolph (9060, USA) mouthpiece and put the accommodating nose clip onto the participants nose. The Cortex analysis would automatically take place from when the turbine detected gas from the participant breathing. Thus, making it the most practical measurement

to take in parallel with the bloods. The CELIF BrAce needed a little more preparation with using the computer to press ‘start’ and then connecting the gas inlet to the CELIF BrAce mouthpiece before the participant started breathing, and then pressing ‘stop’ on the computer to finish the measurement and detach the gas inlet tube from the mouthpiece. The research team also decided to take the Ketonix® measurements last due to being able to switch the CELIF BrAce mouthpiece to the Ketonix® device, whilst connecting it to the Ketonix® app, which required taking the measurement almost immediately, otherwise the device would disconnect from the app.

4.2.3 How the CELIF BrAce detector data was analysed

Following the completion of each trial, the BrAce data from the CELIF BrAce detector was analysed. The BrAce data collected from the CELIF BrAce detector was analysed using a coding software (‘Python’), whereby a Physics Masters student from Durham University created an algorithm using Python to analyse and provide BrAce measurements, which were detected by the CELIF BrAce. After importing the CELIF BrAce files into the software after testing, the files were then transferred into a Microsoft Excel file in a table format.

4.2.4 Blood capillary glucose and betahydroxybutyrate

Blood glucose (Hemocue AB, Sweden) and betahydroxybutyrate (Abbott Freestyle, England) measurements were taken before and during exercise. These measurements were taken from blood capillary samples from the participants fingers. It has been shown that capillary samples are more popular than venous samples with participants when frequent

measurements are needed due to being less painful than venous samples (Wilmore *et al.,* 2016).

4.3 Results

The results in Table 3 and Table 4 highlight the length of time (s) it took to measure each variable. Moreover, Table 5 and Table 6 present CELIF BrAce data from the two trials within non-fasted participants.

Trial 1: Pilot Participant 1 and Participant 2.

Table 3: Pilot participant 1's time taken for measurements during exercise.

	Cortex	**Glucose**	**Betahydroxybutyrate**	**Triglycerides**	**CELIF BrAce**	**Ketonix®**
Time 10	75 s	30 s	30 s	120 s	90 s	30 s
Time 20	75 s	20 s	20 s	90 s	90 s	30 s

Legend: Time 10 = Time (s) it took to analyse each variable at 10 min of exercise. Time 20 = Time (s) it took to analyse each variable at 20 min of exercise.

Table 4: Pilot participant 2's time taken for measurements during exercise.

	Cortex	**Glucose**	**Betahydroxybutyrate**	**Triglycerides**	**CELIF BrAce**	**Ketonix®**
Time 10	70 s	30 s	30 s	120 s	90 s	30 s
Time 20	70 s	30 s	20 s	90 s	300 s	30 s

Legend: Time 10 = Time (s) it took to analyse each variable at 10 min of exercise. Time 20 = Time (s) it took to analyse each variable at 20 min of exercise.

Tables 3 and 4 highlight how long the lead researcher required to collect and analyse each variable. The length of time the lead researcher needed to measure each variable was similar between the two pilots, however the main difference was the time it took to take the CELIF BrAce measurement (TIME 20, CELIF BrAce) due to forgetting to press 'stop' on the CELIF BrAce device.

Table 5: Participant 1's CELIF BrAce measurements when following their usual diet with no restrictions involved.

File	No.Breaths	ETCO2(%)	BrAce(ppm)	Error(ppm)
Measurement 1 - exercise (10 min)	9	8.02	0.85	0.58
Measurement 2 - exercise (20 min)	18	7.89	0.70	0.50

Table 6: Table 6: Participant 2's CELIF BrAce measurements when following their usual diet with no restrictions involved.

File	No.Breaths	ETCO2(%)	BrAce(ppm)	Error(ppm)
Measurement 1 - exercise (10 min)	13	10.33	1.09	0.54
Measurement 2 - exercise (20 min)	15	8.25	0.13	0.58

Tables 5 and 6 show the CELIF BrAce readings from two different participants. The BrAce readings represent suitable data, given what the literature suggests for non-fasting healthy individuals (0-2 ppm, Anderson, 2015). Despite these findings being similar to what the research suggests for non-fasting healthy individuals, the BrAce (ppm) measurement in Table 6, Measurement 2 – exercise (20 mins) is 0.96 ppm lower than the first reading at 10 mins, which is a higher drop off than the reduction highlighted in Table 5. The differences in

readings could be because of the CELIF BrAce not being stopped as previously mentioned, or it could be because the gas inlet line was pushed too far past the breathing hole (see section 4.4.2 for more information).

4.4 Discussion

In summary, the results suggested that it is possible to collect blood measurements (capillary blood glucose and betahydroxybutyrate) at the same time as the $\dot{V}O_2/\dot{V}CO_2$ and Cortex M3B measurements. Following these measurements, the CELIF BrAce was taken, with no issues aside from ensuring the lead researcher remembers stop the measurement correctly and that the gas inlet line is not pushed too far past the breathing hole. After the CELIF BrAce measurements, the Ketonix® measurement process was efficient, providing that the app was already connected and calibrated to the device. If this was not to happen, the measurement time would take longer.

4.4.1 The $\dot{V}O_2/\dot{V}CO_2$, Cortex M3B and bloods

The $\dot{V}O_2/\dot{V}CO_2$ and Cortex M3B measurements were straightforward to take and analyse due to the M3B being able to function automatically when it detects human breath via the turbine. However, due to the difficulty and failure to take triglyceride (Accutrend, Gc, Germany) measurements, the research team decided moving forward that no triglyceride measurements would be taken. The reasons for the difficulty and failure to take valid triglyceride measurements was due to the inexperience of the lead researcher taking triglyceride samples, and the specific technique required to obtain a sufficient triglyceride sample. Overall, not having triglyceride measurements did not affect the overall results of the experimental study because this study is primarily focused upon carbohydrate availability. Additionally, the other

metabolic measurements already involved in the study i.e., BrAce (strong correlation of r = 0.88 with fat metabolism), betahydroxybutyrate concentration and RER provided a strong indication as to what level of fat oxidation was taking place at the time of measuring (Sasaki *et al.*, 2011; Cox and Clarke, 2014; Wilmore *et al.*, 2016). Despite the difficulty obtaining the triglyceride samples, the blood capillary glucose and betahydroxybutyrate samples were taken correctly and effectively in a short period of time (~50 s). Furthermore, the research team discovered it was possible to take and analyse the blood capillary glucose and betahydroxybutyrate samples during the 60 s of the $\dot{V}O_2$ and $\dot{V}CO_2$ cortex measurements. It is important to note that ~10 s was required for the Cortex M3B to detect the participant's breath, which needs to be considered for total amount of time to take the $\dot{V}O_2/\dot{V}CO_2$ measurements. This was highlighted in Tables 3 and 4 where all measurements of capillary blood glucose and ketones were taken ≤ 60 s. This was apparent even when the lead researcher had to re-puncture the participants finger before taking the blood capillary glucose and betahydroxybutyrate measurements due to a lack of blood (see Table 4). After completing the blood measurements, ~20 s wait was required for 60 s of gas analysis to be completed before removing the Hans Rudolph mouthpiece and nose clip, and then removing the saliva and beginning the CELIF BrAce prototype measurements. To save more time, the lab technician recommended to have a supervisor whose role would be to take charge of removing the mouthpiece and nose clip from the participant, to allow the lead researcher to set-up the CELIF BrAce prototype straight after taking the blood capillary glucose and betahydroxybutyrate (see next paragraph). In summary, firstly taking the Cortex M3B and blood measurements in parallel was the most effective sequence from a time and practical standpoint.

4.4.2 The CELIF BrAce

Prior to these pilot tests, the research team discovered that there was a 10-15 s delay for the CELIF BrAce detector to detect any gas. Due to this delay, it was made sure that on the computer screen after pressing "start", the CO_2 levels were at baseline, and when a clicking sound from the machine (immediately after pressing "start") was heard, the gas inlet tube and mouthpiece were then connected. The 60 s of normal breathing then started for the participant; the research team could then track this on the computer (see Figure 15 – comparison of baseline to first breath) and would calculate 60 s from the first breath. As the final breath of the 60 s approached, the researcher team told the participant to take a deeper inhale and then perform and end tidal breath (i.e., deplete the lungs as much as possible). Again, due to the delay of the gas from the participant's breath to reach the CELIF BrAce detector, it was required to wait for the CO_2 line to return to baseline, to ensure that no data points from the 60 s of breathing was lost. During these two pilot trials, the participant could see the computer screen that showed the CO_2 and BrAce measures. After completing the trials, the participants were asked if seeing the live measurements of BrAce impacted their breathing, to which both participants replied that it did, whereby they were trying to breathe harder and faster to try and achieve a higher CO_2 score. From this, it was decided that the participants will not be able to see any of their measures that are involved during exercise to maximise validity (Anderson, 2012). Due to the added time of waiting for the CO_2 to return to baseline, overall, the BrAce CELIF measurements required 90 s to be completed.

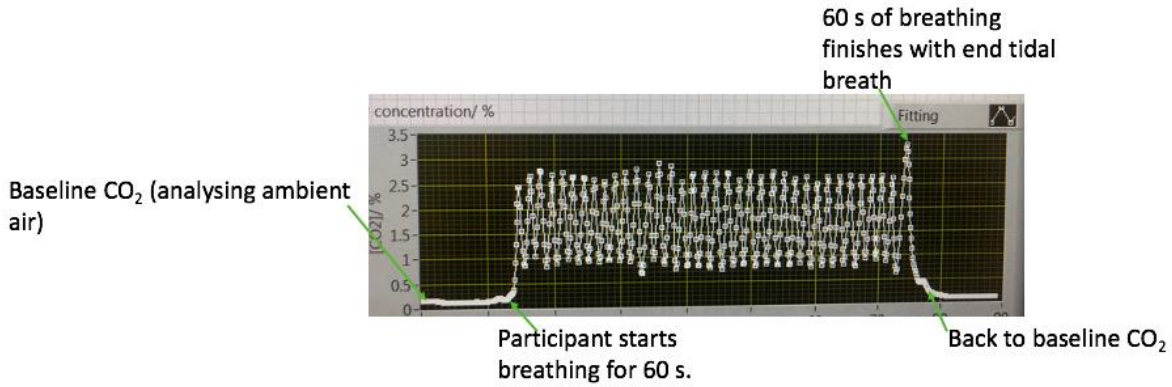

Figure 15: The CO_2 measurements on the CELIF BrAce – baseline vs breathing Figure 15 highlights baseline CO_2 (%) measurements, where the gas inlet line is just exposed to the ambient air for 10-15 s. Then, the gas inlet line is connected to CELIF BrAce mouthpiece before the participant breathes for a minute (CO_2% then rises, with each breath recorded) before performing an end tidal breath (CO_2% reaches its highest reading). After the end tidal breath is performed, the participant removes the gas inlet line from the mouthpiece and the lead researcher stops the measurement after the CO_2% has returned to baseline for 10-15 s.

Following these trials, a couple of other practical considerations that needed to be addressed were discovered. Firstly, the lead researcher forgot to stop the CELIF BrAce measurement 2 - exercise (20 min) (see Table 5), and during the time between removing the gas inlet line from the mouthpiece and stopping the recording of the CELIF BrAce was 3 min. As a result of this, the gas inlet line was taking in the laboratory air for approximately 90 seconds. Furthermore, the detachment of the gas inlet line from the mouthpiece could suggest the anomaly looking CELIF BrAce reading from Table 6, Measurement 2 – exercise (20 min) when comparing the other BrAce reading from Table 6 and the two BrAce measurements from Table 5. This could be because the way in which the data analysis could be coded,

whereby the acetone is taken as an average over the testing period, and due to taking the lab air for a sustained period (where less acetone would be present than in a participant's breath) could explain the low reading. Thus, it was discovered that the analysis should be stopped once the CO_2 has reached baseline. Additionally, for this trial, the lead researcher inserted the yellow gas inlet line past the breathing hole (see Figure 16 for an example) to see if any difference would occur. Consequently, this could also have impacted the measurement due to the gas inlet line taking in less air from the breathing hole. From this, it was ensured that when the participant completed their end tidal breath and the CO_2 returned to baseline, the measurements would be finished. Additionally, moving forward, it was ensured that the gas inlet line was inserted correctly, whereby the top of the gas inlet line, lines up with the breathing hole of the mouthpiece. From doing these two things, the research team thought it would maximise the quality of the CELIF BrAce results.

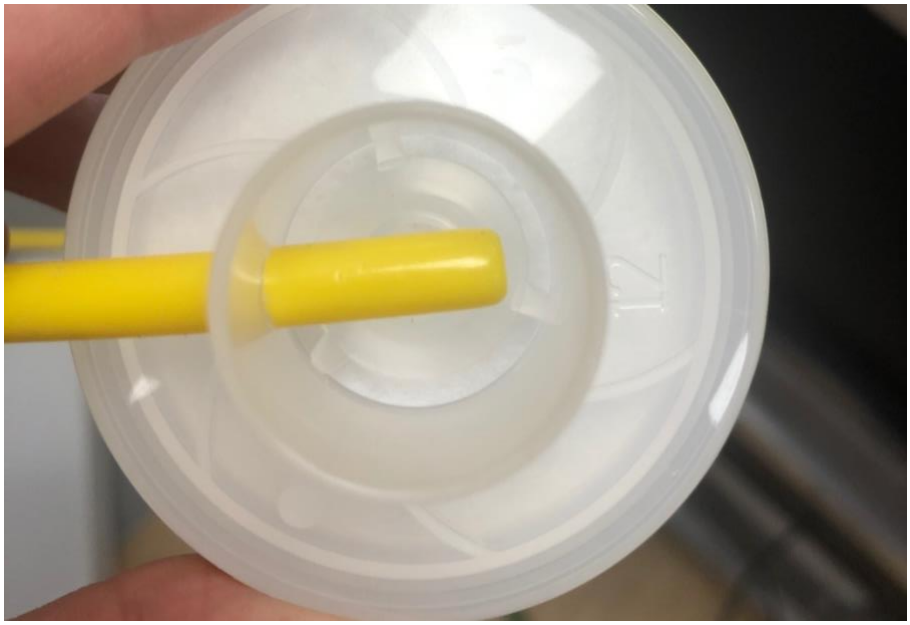

Figure 16: A comparison of the gas inlet line covering and not covering the hole where the acetone from an individual's breath goes. This may impact the amount of the participant's breath goes down the gas inlet line, thus impeding the validity of BrAce detected by the CELIF BrAce detector. Thus, during future data collection, the research team ensured the gas inlet line was just below the hole of the mouthpiece.

4.4.3 The Ketonix®

The Ketonix® was straightforward to use and did not take much time to collect and analyse the data. It is important to note that the research team connected the Ketonix® device to the app via Bluetooth and needed to take the reading almost immediately, otherwise the connection between the app and the device would disappear. To save time, the Ketonix® device was connected the to the app (approximately taking 30 s) whilst the participant was taking their CELIF BrAce measurements. Through applying this protocol, it supported the practicality of taking measurements during data collection.

4.4.4 Positioning of equipment

A visual representation of how the equipment was positioned is shown in Figure 17 and Figure 18. Figure 17 shows where the equipment would be placed if the participant had their blood taken from their left hand. Conversely, if the participant wanted a blood capillary sample to be taken from their right hand, the table that is holding the blood markers would move to the other side of the bike, whereas the rest of the equipment would stay where it is, due to it being in front of the participant. Figure 18 shows how the equipment was set-up on the table.

Figure 17: The decided positioning of the equipment for the research project. The measuring devices were placed to the side of the participant where they chose to have blood taken from. This participant wanted blood taken from their left hand, consequently, if a participant wanted their right hand to be used, the table would be put on the opposite side.

Figure 18: The ordering of blood measurements left-to-right. Firstly, the capillary blood glucose would be taken, followed by the betahydroxybutyrate measurement.

4.4.5 Finalised protocol for the MRes data collection

Following our findings that have been discussed within this chapter, the finalised protocol for the MRes pilot experimental study during the exercise components was:

1) Whilst taking $\dot{V}O_2/\dot{V}CO_2$ from the Cortex (using the Hans Rudolph nose clip and mouthpiece), collect blood glucose and betahydroxybutyrate measurements and analyse (70-80 s). It was recommended that when a minute of data has been collected, a further member of the research team should remove the mouthpiece and nose clip from the participant, whilst wiping the mouthpiece from any excess saliva. This allows the lead researcher to begin taking the CELIF BrAce measurements quicker.
2) Take the participants acetone using the CELIF BrAce detector. Allow time for the delay before and after the participant has been breathing for a minute (breathing

normally for the first 59 s, with the final breath being a deep inhale and an end tidal exhale) (90 s). Whilst the participant completes their 60 s of CELIF BrAce breathing, connect the Ketonix® device to the compatible app (30 seconds).

3) After attaching the CELIF BrAce mouthpiece to the cardboard and tape that is attached to the Ketonix® device, the participant then does one end tidal breath (20 seconds).

In conclusion, the maximum amount of time it took to perform measurements during this testing period was 3 min 20 s, which needed to be completed every 15 min during the experimental pilot data study. Using 15 min time points during exercise is typical because it provides a suitable length of time for physiological adaptations to take place, given the moderate-to-high intensity this experimental pilot trial is using (Wilmore *et al.*, 2016). As found from this chapter, using 15 min within the experimental pilot between measurements provided sufficient time to complete and analyse the measurements, whilst setting up again to recollect the measurements. Finally, having a longer period than 10 min that was used in this chapter for the experimental pilot was instigated by the awareness of the research team, who were conscious that the time it took to complete the measurements during the experimental pilot trail could vary slightly. The timings for completing measurements could have been varied because the participant is exercising at a higher intensity (i.e., 75% $\dot{V}O_{2max}$) than was used within this pilot study, which could have made it more difficult to collect and analyse measurements.

CHAPTER 5 – THE CELIF BRACE PROTOTYPE VS KETONIX®

5.1 Introduction

After identifying the most effective sequence for collecting the required measurements, the research team wanted to compare the two BrAce detectors at rest with non-endurance athletes. This pilot study compared BrAce concentrations between two BrAce devices whilst two healthy non-endurance-trained participants followed three different nutrition strategies. Through being able to compare real-time resting BrAce levels between the CELIF BrAce prototype with a device that is currently in the market, will provide some indication as to how effective the prototype is at detecting the level of carbohydrate following three different nutrition strategies. Furthermore, due to the level of BrAce concentration being individual dependent, and due to the participants being non-endurance based, it will further highlight which BrAce detector is more sensitive to identify such variations between individuals. Furthermore, due to the participants in this chapter being non-endurance, it is fair to assume that their bodies will not be as attuned to fat metabolism or possess a greater metabolic efficiency than endurance athletes. This consequently generates interest to see if the two BrAce detectors can be flexible to detect and accurately specify the variations and characteristics of different individuals (health state, exercise experience etc.) following different nutrition strategies. Additionally, within the Ketonix® app, there is an option box to tick whether or not the participant was following a ketogenic strategy (as shown previously on Figure 7), which provides a further aspect to analyse when analysing the BrAce concentrations between the two devices. The first aim of this trial was to compare the BrAce concentrations measured by the CELIF BrAce prototype (Chemistry Department at Durham University), and a BrAce detector currently on the market (Ketonix®). The second aim of this

trial was to understand the impact on the readings generated from adjusting the Ketonix® app settings.

5.2 Methods

The day before coming into the laboratory to complete measurements on the CELIF BrAce prototype and Ketonix®, the two participants (002 and 003) followed three nutrition strategies. The nutrition strategies were normal diet with no restrictions or fasting, normal diet with > 14 h fast and ketogenic strategy with and without > 14 h fast. Each participant noted down their final meals before entering the laboratory for the lead researcher to analyse their carbohydrate intake using Nutritics Nutrition Analysis Software (Nutritics, Republic of Ireland). The protocol for each device was different. Firstly, the protocol of the CELIF BrAce prototype reflects that of what was previously done during the pilot testing, whereby the participants would breathe for 60 s with their last breath being an end tidal breath. Similarly, for the Ketonix® protocol, the participants performed one end tidal exhale into the device for as long as they could. Whilst taking the measurements, one 60 s measurement was taken from the CELIF BrAce prototype and two were taken from the Ketonix®. The rationale behind taking two measurements using the Ketonix® app was because there was an option box to tick whether or not the participant taking the measurement was following a ketogenic strategy. Finally, to prevent any external influence on their breathing performance during all measurements, the participants were not able to see the devices that were tracking their live BrAce concentrations (Atkinson, 2012).

5.3 Ketonix® in research

Akturk et al. (2021) reported that after over 500 measurements in 19 non-fasting adults a significant association between BrAce and betahydroxybutyrate concentration when using Ketonix® and a betahydroxybutyrate meter (Precision Xtra, Abbott) ($P < 0.005$) was found. This finding reflects what the literature suggests to happen (Musa-Veloso *et al.*, 2002; Španěl and Smith 2011; Anderson, 2015). However, due to the participants being non-fasting, this limits the credibility of the Ketonix® for this study. Within the sport and exercise realm, there was only one research article that used the Ketonix® device (Mujik, 2019). The research used the Ketonix® to analyse the level of BrAce in a long-distance triathlete following a LCHF diet. Although the results of BrAce concentrations were brief; the author highlighted that BrAce levels were "constantly elevated" from following a habitual LCHF diet (p. 8, Mujik, 2019). In turn, suggesting that the Ketonix® measurements are valid to detect BrAce concentrations. Most research using the Ketonix® device was focused around either untrained participants or supporting participants in the detection and management of diabetes ketoacidosis and was posited to be a suitable mechanism to detect BrAce levels (Prabhakar *et al.*, 2014; Akturk *et al.*, 2021; HeadsUpHealth, 2022).

5.4 Results

The results show the differences in BrAce concentrations between the CELIF BrAce prototype and the Ketonix® following each of the three nutritional strategies.

5.4.1 Normal diet with no fast

Participant 002

Last meal: 7 AM (4 h before lab visit) – porridge oats with milk – 45 g carbohydrate

Participant 003

Last meal: 9 AM (4 h before lab visit) – four 50% reduced fat pork sausage sandwiches (2 bagels) with tomato ketchup – 105 g carbohydrate

5.4.2 Normal diet with no fast CELIF and Ketonix® BrAce results

Table 7: The CELIF BrAce measurements of Participant 002 and 003 following a normal diet with no fast.

File	**No.Breaths**	**$ETCO_2$(%)**	**BrAce(ppm)**	**Error(ppm)**
Participant 002	15	9.91	0.64	0.58
Participant 003	15	6.53	0.24	0.41

Table 8: The Ketonix® measurements of Participant 002 and 003 following a normal diet with no fast.

Participant	**Ketonix® acetone concentration (ppm) (ketogenic box unticked)**	**Ketonix® acetone concentration (ppm) (ketogenic diet box ticked)**
002	0	10.9
003	0	39.7

Tables 7 and 8 show the differences between the participants BrAce concentrations when following their 'normal diet'.

The CELIF BrAce prototype readings in Table 7 show readings that are suggested by the literature, due to being in the range of 0-2 ppm, which is widely suggested for individuals following a normal diet (Anderson, 2015). More specifically, participant 002 had a 0.40 ppm higher than participant 003, which could be because participant 003 had 60 g more carbohydrate than participant 002, and the meal of participant 003 was consumed sooner to the analysis (2 h) than participant 003 (4 h).

However, the Ketonix® readings in Table 8 when the ketogenic box was ticked are more ambiguous than the CELIF BrAce measurements. These are BrAce readings which oppose the literature. When the ketogenic diet box was unticked, the Ketonix® provided similar readings to the CELIF BrAce prototype.

5.4.3 Normal diet with > 14 h fast

Participant 002

Last meal: Dinner at 6 pm (17 h before lab visit) – (Salad bowl, scone with clotted cream and jam, 3 scoops of ice cream, caramel wafer bar) - 146 g carbohydrate

Participant 003

Last meal: Dinner at 6 pm (17 h before lab visit) – 4 hot dogs in brioche buns with tomato and barbecue sauce – 130 g carbohydrate

5.4.4 Normal diet with > 14 h fast CELIF and Ketonix® BrAce results

Table 9: The CELIF BrAce measurements of Participant 002 and 003 following a normal diet with > 14 h fast.

File	**No.Breaths**	**$ETCO_2$(%)**	**BrAce(ppm)**	**Error(ppm)**
Participant 002	15	11.69	0.97	0.6
Participant 003	11	7.44	0.29	0.62

Table 10: The Ketonix® measurements of Participant 002 and 003 following a normal diet with > 14 h fast.

Participant	**Ketonix® acetone concentration (ppm) (ketogenic diet box unticked)**	**Ketonix® acetone concentration (ppm) (ketogenic diet box ticked)**
002	0	0
003	0	1.5

The results from Tables 9 and 10 provided BrAce concentrations that reflected what the literature suggests, given the fasted state of the participants and when comparing these results to the literature (Anderson, 2015; Alkedeh and Priefer, 2021).

5.4.5 Ketogenic strategy with and without > 14 h fast

Participant 002 (ketogenic strategy without 14 h fast)

Last meal: Dinner at 8.30 AM (2.5 h before lab visit) – omelette with handful of mushrooms and tomatoes – 5 g carbohydrate

Participant 003 (ketogenic strategy with > 14 h fast)

Last meal: Dinner at 6 pm (17 h before lab visit) –one slice of white bread with cheese mayonnaise salad – 22.5 g carbohydrate

5.4.6 Ketogenic strategy with and without > 14 h fast CELIF and Ketonix® BrAce results

Table 11:The CELIF BrAce measurements of Participant 002 and 003 following a ketogenic strategy with > 14 h fast (Participant 003) or without a > 14 h fast (Participant 002).

File	**No.Breaths**	**$ETCO_2$(%)**	**BrAce(ppm)**	**Error(ppm)**
Participant 002	15	5.44	1.61	0.54
Participant 003	12	5.44	3.33	0.52

Table 12: The CELIF BrAce measurements of Participant 002 and 003 following a ketogenic strategy with > 14 h fast (Participant 003) or without a > 14 h fast (Participant 002).

Participant	**Ketonix® acetone concentration (ppm) (ketogenic diet box unticked)**	**Ketonix® acetone concentration (ppm) (ketogenic diet box ticked)**
002	0	3
003	0	53

The CELIF BrAce presented measurements between the participants that aligned with the research. This was highlighted because Participant 002 who fasted for a shorter period of time than participant 003 had a 1.72 lower ppm (Table 11). The Ketonix® data, however, was more questionable. Although, within the 'ketogenic diet box ticked' column in Table 12

identified that participant 003 was following a more strenuous fasting protocol (highlighted by the 50 higher ppm than participant 002), however the result of 53 ppm is not what you would expect after 24 h of following the ketogenic diet (Anderson, 2015; Alkedeh and Priefer, 2021). Also, when the ketogenic box was unticked, no acetone was detected, which further questions the irregularity of the Ketonix®, especially when considering the differences between the two findings when the boxes were ticked/unticked.

5.5 Discussion

Overall, the results suggest that the CELIF BrAce prototype produced the most findings that resonated with the literature, given the nature of the three nutritional strategies that had various levels of fasting and carbohydrate reduction.

When no ketogenic strategy was implemented by the participants, the CELIF BrAce prototype and the Ketonix® (ketogenic box unticked) produced similar data when comparing to what the literature suggests. Researchers such as Španěl and Smith (2011) and Alkedeh and Priefer (2021) suggested a healthy individual when not fasting or following a ketogenic nutrition strategy should have a BrAce concentration of 0 - 2 ppm. The similarity in findings are highlighted in Tables 7 and 9 for the CELIF BrAce prototype data and Tables 8 and 10 (ketogenic box unticked) for the Ketonix®. Furthermore, the CELIF BrAce prototype presented rational data for the normal diet with > 14 h fast (Table 9) and ketogenic strategy with/without > 14 h fast (Table 11), which was highlighted with the increase in BrAce as fasting/carbohydrate restrictions were introduced, whilst also maintaining comparable data to what the literature suggests (Anderson, 2015). The research has suggested ~2 ppm for a 14 h fast and 2 – 6 ppm for an acute ketogenic strategy (Anderson, 2015; Alkedeh and Priefer, 2021). Moreover, the CELIF BrAce differentiated between the two participants when Participant 2 followed a ketogenic strategy with no fast, and Participant 3 who followed a

ketogenic strategy with a > 14 h fast. This further indicates that the CELIF BrAce is sensitive to detecting varying levels of carbohydrate availability. Additionally, the Ketonix® BrAce measurements did not increase ('ketogenic diet' box unticked) during the normal diet with no fast trial (Table 12). Furthermore, the Ketonix® BrAce concentration readings did increase for both participants when they followed the ketogenic strategy with/without a fast (see Table 12, 'ketogenic diet' box ticked). Although the BrAce concentrations from both devices followed the correct pattern, the CELIF BrAce prototype presented BrAce data that is more aligned to the literature than the Ketonix® device.

5.5.1 High variability with Ketonix®

Despite the Ketonix® generally presenting findings that follow an expected pattern when individuals are either fasted or non-fasted, the variability in scores when ticking and unticking the ketogenic box questions the effectiveness of the Ketonix® device for detecting carbohydrate availability. Aside from the normal diet with > 14 h fast trial, the variability in the other two trials (Tables 8 and 12) highlight the Ketonix® is programmed in such a way to naturally suggest higher scores when you tick the 'following a ketogenic box' (see Figure 19). Even for the ketogenic strategy with > 14 h fast trial, the score of 53 ppm would be a score you would expect from an individual who is healthy and has fasted ≥ 3 days and not from an individual who is following an acute ketogenic strategy (Anderson, 2015). Thus, suggesting that the Ketonix® is not providing a true reading. Furthermore, a container of liquid acetone was held next to the Ketonix® device (see Figure 20) with the 'ketogenic diet' box unticked to see what score the device would give, and the Ketonix® app presented a score of 0 ppm. This shows that the Ketonix® device did not detect any acetone and indicated that regardless of your ketogenic state the Ketonix® may not provide a true BrAce reading. Finally, there were a series of automatic updates that took place within the Ketonix® app,

which seemed to provide stricter (lower) measurements. This could provide an explanation for the wide variety of BrAce readings reported in this chapter from the Ketonix®. In summary, the Ketonix® provided a wide variability in BrAce concentrations, with BrAce measures not reflecting the ketogenic state of the participants, especially when ticking the ketogenic box. This continuous development of the app further suggests that the Ketonix® requires more development to provide BrAce readings, which are more closely related to what the literature suggests and the ketogenic state of the participant.

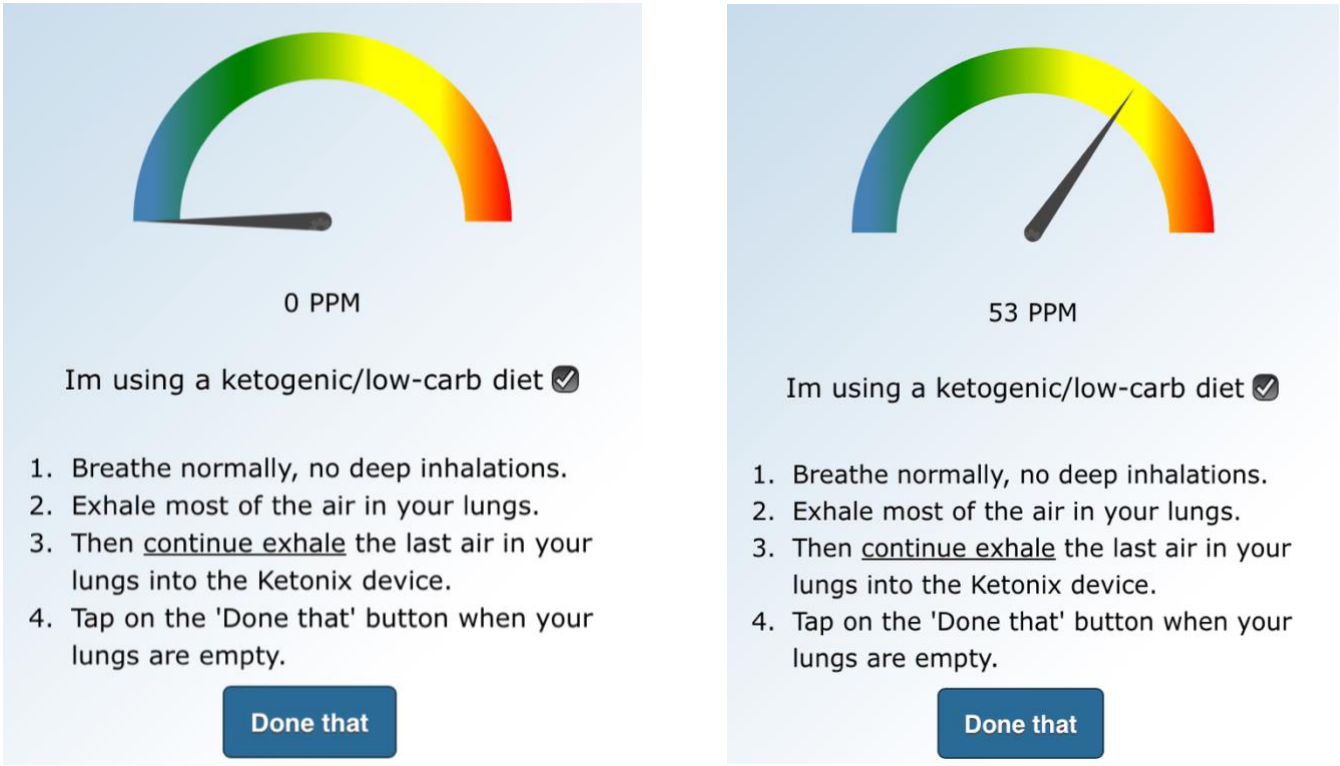

Figure 19: The comparison of Ketonix® BrAce values when ticking/unticking ‘ketogenic diet’ button. The differences in BrAce concentrations highlights the large variability in values that the Ketonix® provided.

Figure 20: The Ketonix® placed over a container of liquid acetone to further ascertain the level of BrAce concentration variability the Ketonix® provides.

CHAPTER 6 – EXPERIMENTAL PILOT

6.1 Introduction

Following establishing the most efficient procedure for collecting the gas samples, identifying the most effective sequence to collect the measurements required, and understanding the comparisons between the two BrAce detectors, the research team were ready to complete an experimental pilot with an endurance athlete. Additionally, the research team aimed to explore whether the agreed protocol from Chapter 4 was practically effective with endurance athletes during exercise. Ethical approval was obtained from the Durham University Department of Sport and Exercise Sciences Ethics Committee. The participant (n=1) was provided with a detailed description of the study's testing protocols to familiarise themselves prior to data collection. The participant provided informed consent before taking part in the study. The aim of this experimental pilot was to explore whether the agreed protocol for the experimental pilot study was practically effective with endurance athletes during exercise.

6.2 Participants

Endurance athletes were targeted to be recruited for the pilot of the protocol. An ultra-endurance athlete was recruited to take part in the pilot of the protocol and a qualitative interview. The inclusion criteria for this present study was that the participant needed to be injury free, male or female, aged between 18-40 years and engage in endurance exercise > 8 h per week. The inclusion criteria yielded data sets from one ultra-endurance athlete (male, ultra-endurance, running; age: 38 years; stature: 1.76 m; body mass: 66.1 kg).

6.3 Methods

6.3.1 Baseline testing

The procedure within this research study is depicted in Figure 21. The Participant completed a 3-day food and activity log prior to attending a baseline visit and was asked to maintain their habitual intake and training throughout the duration of the study. At a baseline visit, prior to the experimental trials commencing, the participant completed a health screening questionnaire (PAR-Q, ACSM, 2013, see Appendix 1), an energy availability questionnaire (adapted from Keay *et al.*, 2018, see Appendix 2), had their body mass (Seca 882 scales, England) and stature (Seca 217 stadiometer, England) measured, and provided a capillary blood sample, which was analysed for glucose (Hemocue AB, Sweden) and betahydroxybutyrate (Abbott Freestyle, England). A baseline BrAce (CELIF BrAce prototype, Chemistry Department, Durham University; Ketonix®, Varberg, Sweden) and breath by breath gas analysis (Cortex M3B, Germany) and heart rate (Polar H10, Finland) measures were collected over a resting 10-minute period. Following these initial measurements, the participant completed a $\dot{V}O_{2max}$ test on a cycle ergometer (using the Cortex M3B), which informed the workload for the experimental trials. The seat placement that was chosen by the participant at baseline was standardised for the remainder of the trials. The participant was randomly assigned an order of the four experimental trials by ordering the numbers one to four, with each number representing a different trial. The number that represented each trial was blinded from the participant. The participant started all trial protocols in a rested stated (>24 h training) following a >14 h overnight fast. Food guidance based on the participant's food diary was provided by the lead researcher before each trial using the Nutritics Nutrition Analysis Software (Nutritics, Republic of Ireland). All food guidance was based on the ketogenic strategy, with carbohydrate intake being ≤ 50 g and

protein intake being ~ 1.5 g·kg·BM^{-1} (Sansone *et al.*, 2018). There were ≥ 5 days between trials and where possible the participant was tested on the same day each week.

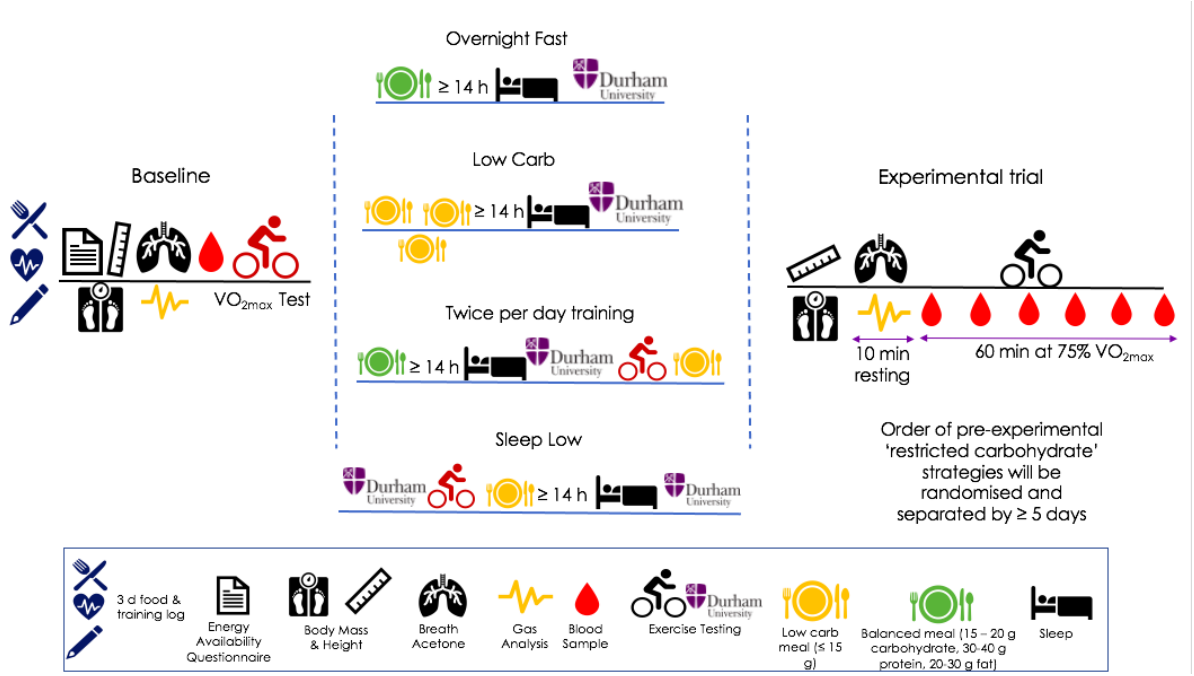

Figure 21: Schematic depiction of the methodology used in this pilot study.

6.3.2 Experimental trial

On arrival, stature and body mass were recorded followed by the completion of a 10 min BrAce and gas/heart rate measurement (to measure $\dot{V}O_2$ consumption and $\dot{V}CO_2$ production) at rest. A baseline capillary blood sample was collected to analyse blood capillary glucose and betahydroxybutyrate. The participant then began a 60 min endurance training session at 75% $\dot{V}$O2max on the cycle ergometer. Breath measurements for acetone and $\dot{V}O_2/\dot{V}CO_2$ were collected every 15 min. Heart rate, rate of perceived exertion (RPE, Borg, 1982), blood glucose and betahydroxybutyrate were also measured every 15 min. The preparation prior to each trial differed and was designed to replicate typical 'train low' strategies, each is outlined below (see Impey *et al.*, 2018 for further information). For all trials, the participant was allowed to consume water ad libitum. After each trial, the participant's body mass was

measured again to allow the lead researcher to recommend how much liquid needed to be consumed for the participant to rehydrate adequately. This replicated the study of Morton (*et al.*, 2009) who recommended total liquid to consume = difference in pre- and post-body mass X 1.5 to provide a value in L to allow the participant to adequately rehydrate.

6.3.3 Qualitative interview

This present study is a method-focused project, and consequently qualitative data was collected in addition to quantitative data to understand the participant's perspective on their experiences during the project. Gaining the participant's perspective on their experiences was implemented to aid the development of the final protocol, specifically around creating the optimal experience for participants. A semi-structured interview was conducted with the participant. The interview was guided by an interview guide (see Appendix 3) with further questions asked based on the participant's responses. A semi-structured interview was chosen rather than a structured or unstructured interview because a semi-structured interview is the most effective at gathering information, due to its flexible nature, thus supplementing the collection of objective data (Kvale and Brinkman, 2009; Guest and Namey, 2013). The interview was conducted by IE (the lead researcher) and took place online via Microsoft Teams and lasted 15 min. The audio transcription was downloaded and used to further analyse. No formal analysis techniques were undertaken given the sample size (n=1); thus, the transcript was studied by IE to note key aspects (i.e., experiences and thoughts during the experimental process) to inform the development of the final protocol.

6.3.4 Train low strategies

Overnight fast: The participant was asked to consume their final balanced meal of the day > 14 h prior to their laboratory arrival time the next day.

Low carb diet: The participant was guided to consume low carbohydrate meals (each meal ~15 g of carbohydrate with a total of 45 $g \cdot d^{-1}$) the day prior to the laboratory visit.

Twice per day training: The participant ate their guided food the day before attending the laboratory where they completed a training session on the cycle ergometer designed to deplete glycogen stores. The participant then consumed a low carbohydrate meal (Arla Skyr natural yogurt, 375 g; 15 g carbohydrate, 39.75 g protein, < 0.5 g fat) before completing the training session as described above with the other trials.

Sleep low: The day prior to the trial, the participant ate all but their evening meal before attending the laboratory where they completed the glycogen depleting cycling training session. The participant then consumed a low carbohydrate evening meal before the participant completed the experimental trail on the following day.

6.3.5 The $\dot{V}O_{2max}$ test

A $\dot{V}O_{2max}$ test was used to determine the aerobic capacity of the participants and to determine the workload for the 75% $\dot{V}O_{2max}$ experimental trials. Following an adapted RAMP protocol of Taylor (*et al.,* 2013), the athlete warmed-up for 5 minutes at 50 W at a cadence corresponding to 60 $revs \cdot min^{-1}$ on a cycle ergometer (Lode, Excalibur Sport, Netherlands). Immediately after the warm-up concluded, the participant proceeded onto the RAMP protocol, with the starting intensity being 60 W at a minimum of 60 revs·min-1, with the power increasing by 30 W every two minutes until the participant fell below 60 $revs \cdot min^{-1}$ at a given absolute intensity. The participants $\dot{V}_E$, $\dot{V}O_2$ and $\dot{V}CO_2$ was measured using the breath-by-breath gas analyser (Cortex, M3B, Germany). The test was terminated when the participant consistently fell below 60 $revs \cdot min^{-1}$ for 5 s. Following the completion of the $\dot{V}O_{2max}$ test, the final min of the test was analysed by taking the average of $ml \cdot min \cdot kg^{-1}$ of

$\dot{V}O_2$ to determine the participant's $\dot{V}O_{2max}$. Subsequently, the corresponding workload (i.e., W) to 75% of the participants' $\dot{V}O_{2max}$ was used for the experimental trials.

6.3.6 Glycogen depletion protocol

The glycogen depletion protocol aims to deplete glycogen from type I and type II muscle fibre types. The warm-up and glycogen depletion protocol replicated the practice of Ruby et al. (2005). The protocol was performed on a Lode ergometer (Excalibur Sport, Netherlands). At the beginning of the glycogen depletion protocol the participant completed a 10 min warm-up at a workload at 55% of $\dot{V}O_{2max}$. The warm-up was performed at a cadence of 60-75 revs·min^{-1}. Immediately following the warm-up, participants completed 10 intervals of 2 min at an intensity of 80% $\dot{V}O_{2max}$, followed by 4 min at 50% $\dot{V}O_{2max}$. Following the 10 intervals, the participant completed 8 min at 60% $\dot{V}O_{2max}$ before finishing the protocol with 12 min at 50% $\dot{V}O_{2max}$. All the workloads following the warm-up were performed at a cadence of ≥ 60 revs·min^{-1}. Prior to completing the protocol, the participant was warned If the cadence consistently dropped (> 5 s) below 60 revs·min^{-1} the protocol would be terminated. Following the completion of the protocol, the participant completed 5 minutes of unloaded pedalling.

6.4 Results

6.4.1 Baseline

Time of last meal/s: 7 AM and 11:30 AM (4 hr 30 min and 30 min before visit). Two pieces of wholemeal toast with 40 g peanut butter and Science in Sport gel (32 g and 22 g carbohydrate).

Total 54 g carbohydrate.

Table 13: Baseline physiological profile of measures after following a 'normal diet' at rest.

Physiological component	**Value**
Blood glucose	7.9 mmol·L
Betahydroxybutyrate	0.0 mmol·L
CELIF BrAce	0.82 ppm
Ketonix® BrAce	0.00 ppm
RER	0.86
Average heart rate	88 bpm
$\dot{V}O_{2max}$ and workload attained	54 ml·min·kg^{-1} and 330 W
75% $\dot{V}O_{2max}$ and corresponding workload	40.5 ml·min·kg^{-1} and 225 W
RER at termination of $\dot{V}O_{2max}$	1.17
Heart rate at termination of $\dot{V}O_{2max}$	191bpm

Following these baseline results, the workload for the participant during the 75% $\dot{V}O_{2max}$ trials was decided.

6.4.2 Twice Per day training

Visit 1: Glycogen depletion protocol

Time of last meal: 6:30 PM (16 hr 30 min before visit), pasta bolognese (21 g carbohydrate)

No measures taken before or during the glycogen depletion protocol.

Visit 2: 75% VO2max protocol

Time of last meal: 1:30 PM (90 min before visit), Skyr natural yogurt (15 g carbohydrate)

Table 14: Physiological measures at rest and during the 'twice per day training' protocol.

Physiological component	**Value at rest**	**Value at 15 min**
Blood glucose	5.6 mmol·L	3.5 mmol·L
Betahydroxybutyrate	0.7 mmol·L	0.2 mmol·L
CELIF BrAce	4.13 ppm	3.25 ppm
Ketonix® BrAce	1.50 ppm	0 ppm
RER	0.74	0.96
Average heart rate	101 bpm	190 bpm
RPE	N/A	15 (hard)

RER = respiratory exchange ratio, RPE = rate of perceived exhaustion (Borg, 1982).

The participant fell consistently below 60 rpm after 17 min. Consequently, the protocol was terminated at 17 min. As exercise progressed, all measurements increased, aside from blood glucose, CELIF and Ketonix® BrAce and betahydroxybutyrate concentrations, which decreased by 2.1 mmol·L, 0.88 ppm, 1.50 ppm and 0.5 mmol·L.

6.4.3 Overnight fast

Time of last meal: 7 PM (17 h before visit to lab), pasta bolognese (21 g carbohydrate)

Table 15: Physiological measures at rest for the 'overnight fast' protocol.

Physiological component	**Value at rest**	**Value at 15 min**	**Value at 30 min**
Blood glucose	4.3 mmol·L	5.2 mmol·L	7.6 mmol·L
Betahydroxybutyrate	0.4 mmol·L	0.2 mmol·L	0.2 mmol·L
CELIF BrAce	4.08 ppm	Technical error	Technical error
Ketonix® BrAce	1.1 ppm	0 ppm	1 ppm
RER	0.75	1.03	1.10
Average heart rate	66 bpm	183 bpm	186 bpm
RPE	N/A	13 (somewhat hard)	18 (very hard)

RER = respiratory exchange ratio, RPE = rate of perceived exhaustion (Borg, 1982).

The participant fell consistently below 60 rpm after 30 min. Consequently, the protocol was terminated at 30 min. As exercise progressed, all measurements increased apart from the CELIF and Ketonix® BrAce and betahydroxybutyrate concentrations, which decreased.

6.4.4 Low carbohydrate

Time of last meal: 7 PM (17 h before visit to lab), pasta bolognese, (21 g carbohydrate)

Table 16:Physiological measures at rest for the 'low carbohydrate' protocol.

Physiological component	**Value at rest**	**Value at 15 min**	**Value at 30 min**	**Value at 45 min**
Blood glucose	4.6 mmol·L	5.8 mmol·L	5.8 mmol·L	8.0 mmol·L
Betahydroxybutyrate	0.3 mmol·L	0.1 mmol·L	0.1 mmol·L	0.2 mmol·L
CELIF BrAce	3.15 ppm	1.64 ppm	Technical error	Technical error
Ketonix® BrAce	2.6 ppm	0 ppm	0 ppm	0 ppm
RER	0.73	1.00	1.04	Technical error
Average heart rate	66 bpm	176 bpm	184 bpm	Technical error
RPE	N/A	14 (somewhat hard)	15 (hard)	19 (very, very hard)

RER = respiratory exchange ratio, RPE = rate of perceived exhaustion (Borg, 1982).

The participant fell consistently below 60 rpm after 45 min. Consequently, the protocol was terminated at 45 min. As exercise progressed, all measurements increased apart from the CELIF and Ketonix® BrAce, and betahydroxybutyrate concentrations, which decreased.

6.4.5 Sleep low

Time of last meal: 7PM (17 h before visit to lab, pasta bolognese, 15 g carbohydrate)

Table 17: Physiological measures at rest for the 'sleep low' protocol.

Physiological component	**Value at rest**	**Value at 15 min**	**Value at 30 min**
Blood glucose	4.1 mmol·L	4.3 mmol·L	4.5 mmol·L
Betahydroxybutyrate	0.6 mmol·L	0.2 mmol·L	0.2 mmol·L
CELIF BrAce	2.12 ppm	3.97 ppm	3.36 ppm
Ketonix® BrAce	0 ppm	0 ppm	0 ppm
RER	0.73	0.95	1.04
Average heart rate	66 bpm	178 bpm	188 bpm
RPE	N/A	16 (hard)	19 (very, very hard)

RER = respiratory exchange ratio, RPE = rate of perceived exhaustion (Borg, 1982).

The participant fell consistently below 60 rpm after 30 min, consequently the protocol was terminated at 30 min. As exercise progressed, all measurements increased apart from betahydroxybutyrate concentrations, which decreased.

6.4.6 Metabolic comparisons between trials

The metabolic measurements between the trials from Tables 13-17 are shown below. The metabolic measurements that were compared were the capillary blood glucose, betahydroxybutyrate, CELIF BrAce and Ketonix® concentrations at rest and during exercise.

Capillary glucose concentrations

The level of capillary glucose concentrations were measured at rest and during 15 min periods during all trials (see Figure 22).

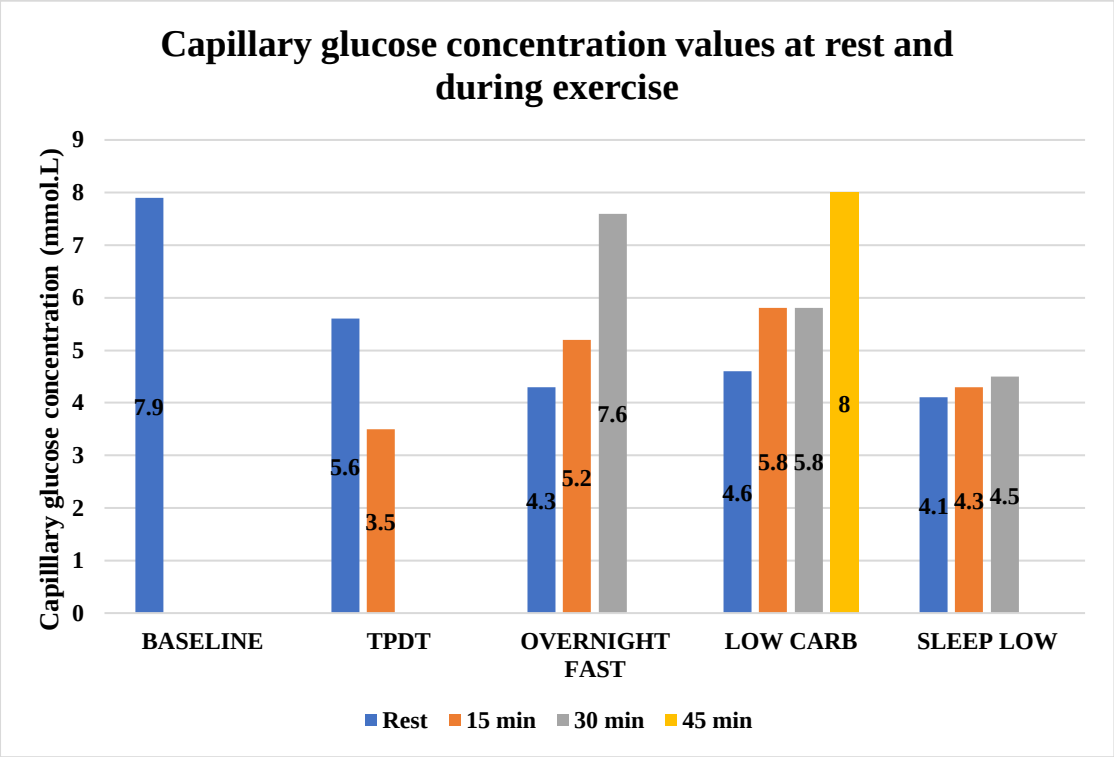

Figure 22: Capillary glucose data presented across all the trials during 15 min regular periods until the trial terminated. TPDT= ‘twice per day training’, LOW CARB= ‘low carbohydrate’.

Betahydroxybutyrate concentrations

The level of betahydroxybutyrate concentrations were measured at rest and during 15 min periods during all trials (see Figure 23).

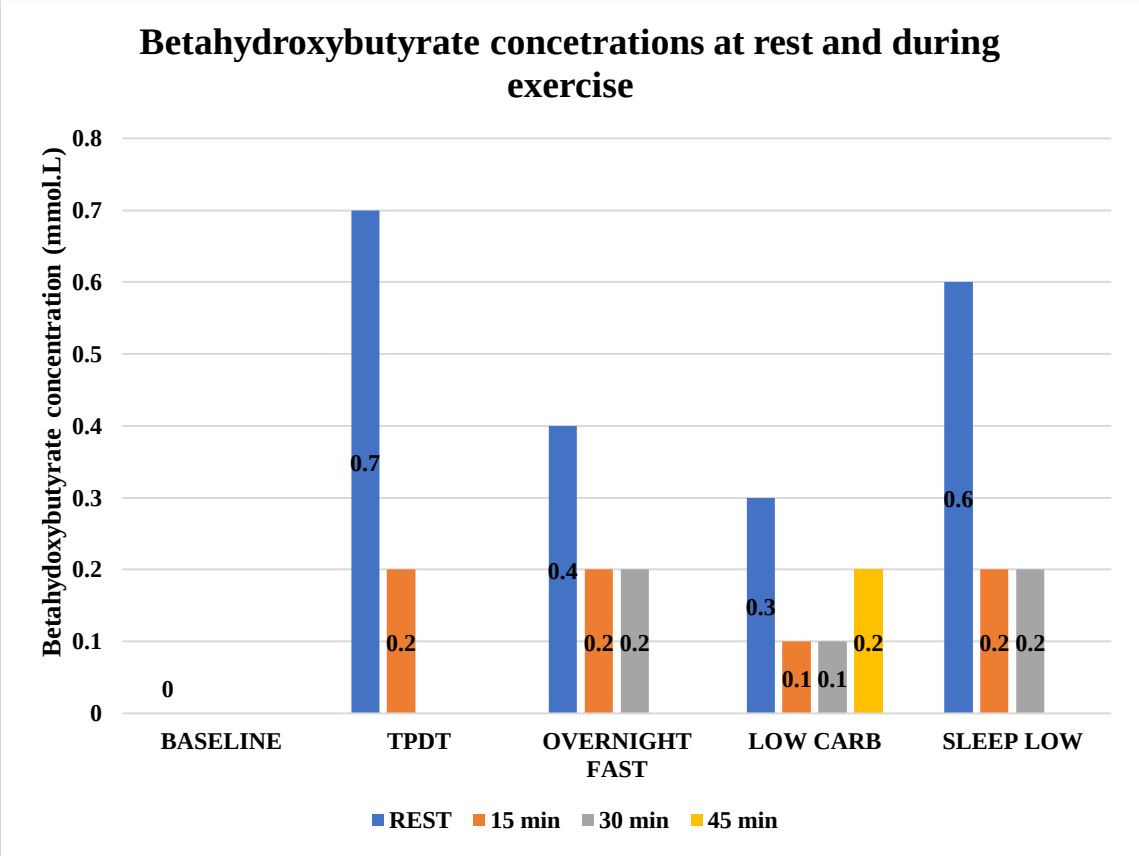

Figure 23: Capillary glucose data presented across all the trials during 15 min regular periods until the trial terminated. TPDT= ‘twice per day training’, LOW CARB= ‘low carbohydrate’.

The CELIF BrAce concentrations

The level of the CELIF BrAce concentrations were measured at rest and during 15 min periods during all trials (see Figure 24).

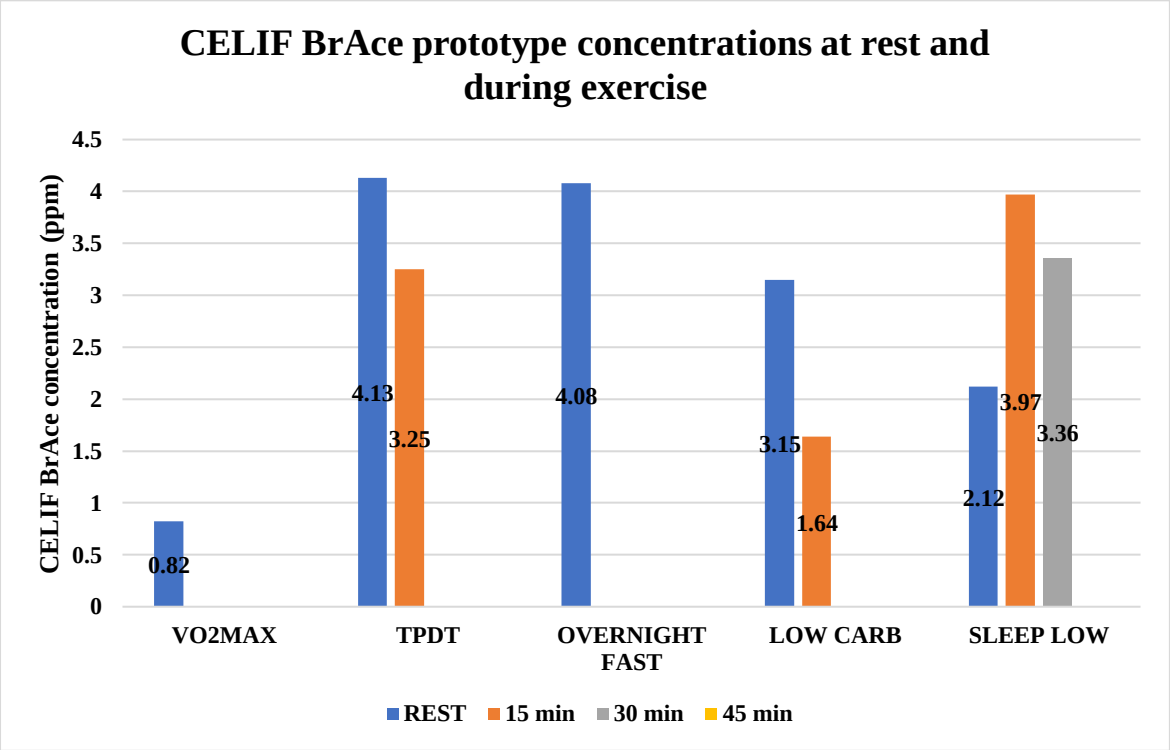

Figure 24: CELIF BrAce data presented across all the trials at rest and during 15 min regular periods until the trial terminated. TPDT= 'twice per day training', LOW CARB= 'low carbohydrate'.

Ketonix® BrAce concentrations

The level of the Ketonix® BrAce concentrations were measured at rest and during 15 min periods during all trials (see Figure 25).

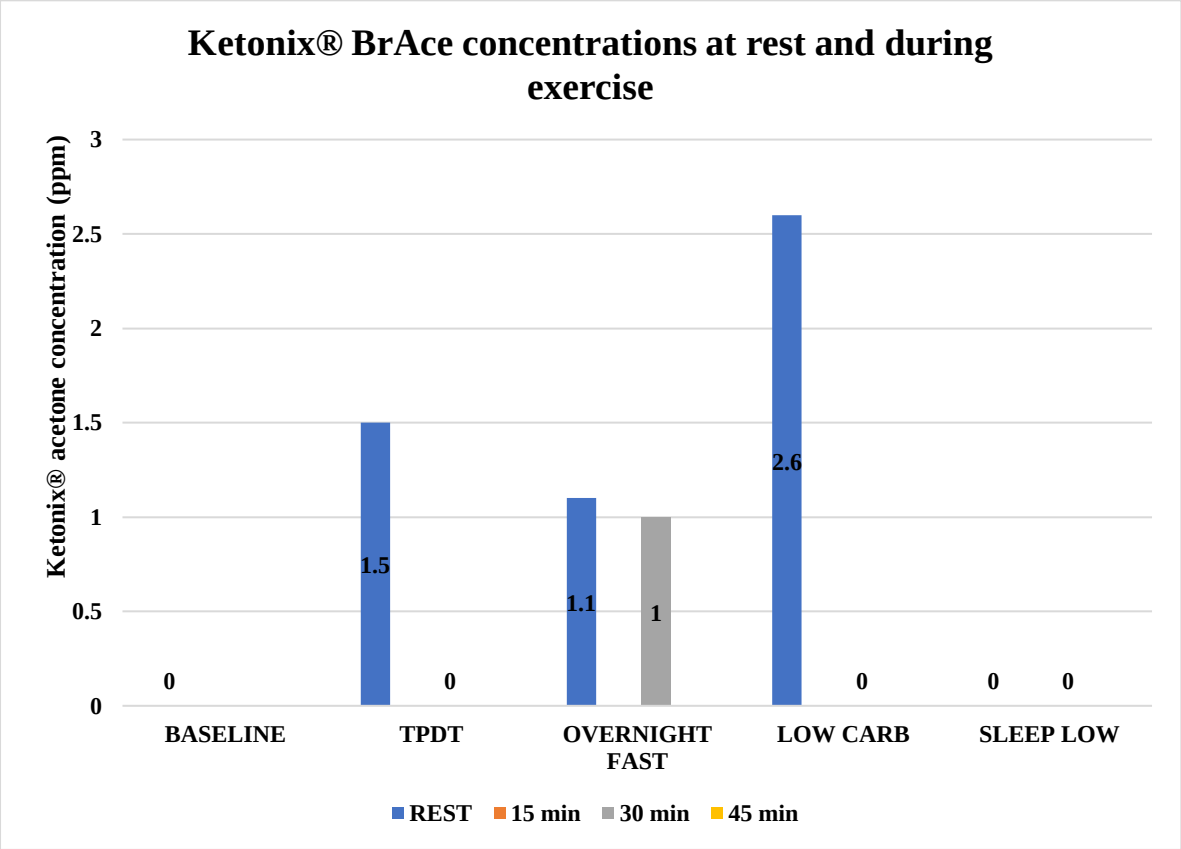

Figure 25: Ketonix® BrAce data presented across all the trials during 15 min regular periods until the trial terminated. TPDT=’twice per day training’, LOW CARB=’low carbohydrate’.

6.4.7 Qualitative feedback

Overall, the participant enjoyed the pilot experimental trials and said they would look to implement “train low” strategies into their training programme after understanding the scientific rationale behind the strategies. The participant did not have any previous

experience of following a ketogenic or reduced carbohydrate strategy and had limited experience at exercising at intensities that were used in these trials (i.e., 75% $\dot{V}O_{2max}$). The participant mentioned that other than feeling weaker from a physical standpoint (which is expected given the carbohydrate reduction) they did not feel negative effects to their physical and psychological health. Furthermore, the participant felt that they had become accustomed to the ketogenic strategy, whereby it did not negatively affect their day-to-day working life. The only trial the participant reflected on with disturbance, was after the second session of the 'twice per day training' protocol. Symptoms included feeling "ill", drained of energy and not being able to concentrate effectively with their work and family duties. This finding is unsurprising given that this is the "train low" strategy that had the highest workload, with the least recovery time.

At the beginning of the pilot experimental protocol, the participant struggled with elements of the equipment, however over time they recalled that they became accustomed to the majority of the equipment and felt comfortable using the apparatus during exercise. The two elements of equipment that raised concern with the participant was the Hans Rudolph 9060 mouthpiece and the Ketonix®. The Cortex 9060 mouthpiece did become more comfortable for the participant as the experimental trials continued, however the increased struggle to breathe (in comparison to the CELIF BrAce mouthpiece) and the build-up of excess saliva were the causes of the Hans Rudolph mouthpiece feeling uncomfortable. Additionally, the excess volumes of saliva could have impacted the gas analysis measurements, which needs to be considered by the research team moving forward. Secondly, the Ketonix® was the most uncomfortable equipment for the participant to use during exercise. Due to the protocol of the Ketonix® involving one end tidal exhale, as the exercise became more difficult for the participant, the end tidal exhale became increasingly demanding to complete, with the

participant noting feelings of dizziness, nausea and an inability to continue exercising at the same workload prior to completing the Ketonix® measurement. Thus, the procedure for obtaining BrAce levels using the Ketonix® became difficult for the participant to complete and resulted in the decrease of physical and psychological performance after the measurement was taken.

6.7 Discussion

In summary, the results from this pilot highlighted two main findings. Firstly, the CELIF BrAce measures at rest and during exercise indicated that the prototype could detect varying levels of carbohydrate availability between the "train low" strategies, albeit some of the BrAce levels did not reflect the other metabolic measurements. This was similar to the Ketonix®, however it detected varying carbohydrate availability levels to a lesser extent. The second main finding was that the participant failed to complete any of the 60 min trials following the "train low" strategies.

6.8 Detecting variations in carbohydrate availability

The CELIF BrAce was able to identify the highest carbohydrate availability of the four trials at the baseline visit. The BrAce and betahydroxybutyrate measurements reported the lowest concentrations at rest of the baseline visit (0.82 ppm and 0.0 mmol·L). This was an expected finding due to the participant following their 'normal' diet, which included the highest level of carbohydrate (54 g) out of all the final meals before each trial started. Furthermore, the higher carbohydrate availability was reflected in the resting capillary glucose concentration (7.9 mmol·L) and RER (0.88) values being the highest out of all the trials. Additionally, the Ketonix® reported the baseline visit to have the joint lowest acetone concentration (0 ppm) with the 'sleep low' trial. Consequently, both BrAce detectors were able to distinguish the

diet with the highest carbohydrate availability, which was supported by the values reported from the additional measures.

For the ‘twice per day training’ protocol, the CELIF BrAce prototype produced BrAce readings that reflected the other metabolic measurements more than the Ketonix® device. The highest resting BrAce value by the CELIF prototype was the ‘twice per day training’ protocol, with a score of 4.13 ppm. The ‘twice per day training’ protocol having the highest resting BrAce concentration appears accurate due to the participant completing a glycogen depleting protocol 3 h before the 75% $\dot{V}O_{2max}$ protocol. Completing the glycogen depletion protocol before the 75% $\dot{V}O_{2max}$ protocol would deplete glycogen stores, thus having a greater reliance on fat metabolism, and therefore ketone metabolism. This also highlights why the betahydroxybutyrate concentration was also at its highest at rest within the same trial (0.7 mmol·L). Thus, it appears the CELIF BrAce prototype identified the lowest carbohydrate availability found within this trial, which had the most appropriate rationale for reporting the highest BrAce levels of the four “train low” strategies. However, this did not reflect the capillary glucose concentration and RER value at rest within the ‘twice per day training’ trial. For capillary glucose concentration, the ‘twice per day training’ protocol presented the highest concentration (5.6 mmol·L) after the baseline blood glucose concentration. These findings are not intuitive as the ‘twice per day training’ trial presented the highest BrAce reading, which suggests that the participant is relying more on fat metabolism. The reason why this result happened, could be because of gluconeogenesis. Due to the participant being depleted of glycogen after the glycogen depletion protocol, the cells created ATP through ketones, glycerol, FAs and amino acids to generate glucose (Burke *et al.*, 2011; Mougios, 2020). The reliance on non-carbohydrate sources would also explain why the capillary glucose decreased during exercise to 3.5 mmol·L, which was the only decrease in capillary

glucose that occurred within all the “train low” strategies. Additionally, due to the high possibility that ketones were responsible for providing energy during this trial, could also justify the decrease in CELIF BrAce (0.88 ppm), Ketonix® (1.50 ppm) and betahydroxybutyrate from 0.7 to 0.2 mmol·L. In summary, the ‘twice per day training’ protocol was suggested to have the most severe depletion in liver and muscle glycogen due to this trial having the highest workload, which resulted in an increased reliance on alternative sources of carbohydrate as fuel, consequently explaining why this trial presented the highest BrAce resting value.

In contrast to the CELIF BrAce prototype, the Ketonix® reported the ‘twice per day training’ protocol to have the second highest BrAce reading, behind the ‘low carbohydrate’ strategy. Due to the participant completing the glycogen depletion protocol before taking this resting value within the ‘twice per day training’ protocol, it could be suggested that the CELIF BrAce provided the more accurate BrAce reading.

For the remainder of the trials, the BrAce detectors presented varying BrAce concentrations. The reasoning behind the differences could be because of a variety of reasons (Bartlett *et al.,* 2013; Bartlett *et al.,* 2015; Impey *et al.,* 2018). Firstly, the updates of the two BrAce detectors could have caused the differences in BrAce concentrations. Specifically, the CELIF BrAce required regular recalibrations for safety and practical reasons. Such recalibrations led to changes within the machine towards the end of data collection, which included the replacement of the CO_2 sensor, which has shown to adapt BrAce readings slightly (Anderson, 2015; Alkedeh and Priefer, 2021). Additionally, the Ketonix® had a series of updates through the data collection period, which could have resulted in stricter measures being reported

(such as those in Chapter 5). Also, the participant's diet the days before following the ketogenic strategy was not tracked. Despite the participant being told to follow their 'typical' diet, the participant may have consciously adapted their carbohydrate intake to compensate the reduced carbohydrate intake from the ketogenic strategy. This in turn, could explain the variance in BrAce concentrations, which are further discussed below.

From the reported BrAce concentrations from both BrAce detectors, it was suggested that the 'sleep low' protocol had either the second (CELIF BrAce) or joint lowest (Ketonix®) BrAce concentration values at rest. During exercise, the CELIF BrAce measurement increased by 1.85 ppm. This was the only trial where there was an increase in BrAce from rest. This finding did not support the other measurements that were taken, for instance, capillary glucose and RER increased. The reason for this finding may be because of the calibration and replacement of the CO_2 sensor that took place before the 'sleep low' trial, which has shown to adapt BrAce readings slightly (Anderson, 2015; Alkedeh and Priefer, 2021). Additionally, during exercise of the 'sleep low' trial, the concentration of betahydroxybutyrate decreased, which could be because some of the betahydroxybutyrate was converted into acetone to be exhaled via the breath (Mougios, 2020). However, the idea that the decrease in betahydroxybutyrate is the reason for the increase in CELIF BrAce does lack support, due to other trials following the same pattern in betahydroxybutyrate concentration, whilst CELIF BrAce continued to decrease. Overall, the 'sleep low' trial did present BrAce measures that were not supported by the other metabolic measurements, which could be because of the adaptations with the CELIF BrAce machine or the decrease in betahydroxybutyrate.

The 'low carbohydrate' trial presented the third highest carbohydrate availability after the 'baseline' and 'sleep low' trial at rest and during exercise, with a CELIF BrAce score of 3.5

ppm (see Figure 24). Interestingly, this was supported with the third highest reported capillary glucose and betahydroxybutyrate concentration at rest as shown in Table 15. This highlights that the BrAce reading reflects the measures of the other metabolic variables. As exercise progressed, BrAce values from both devices and betahydroxybutyrate decreased, which is expected given the increase in blood glucose and RER. Dissimilarly to the CELIF BrAce, the Ketonix® presented the highest carbohydrate availability out of all the trials, including baseline, which did not reflect what the other measurements were suggesting. The BrAce concentrations reported by the Ketonix® further questions its effectiveness for assessing BrAce during exercise. In summary, for the 'low carbohydrate' trial, the CELIF BrAce reported concentrations that were reflective of the other metabolic values, consequently indicating that it is effective at detecting low carbohydrate availability.

The 'overnight fast' trial had the second highest CELIF BrAce measure, suggesting this trial had the second lowest carbohydrate availability. Similarly, to the 'low carbohydrate' trial, the capillary glucose concentration supported the CELIF BrAce measure. The reported capillary glucose concentration within the 'overnight fast' trial was the second highest, which accompanies the second lowest CELIF BrAce reading reported in the same trial. However, despite the CELIF BrAce and capillary glucose measures supporting each other, the betahydroxybutyrate was third highest, which contradicts what would be expected given the previous two measures. Consequently, this contrast in findings questions the effectiveness of the CELIF BrAce prototype. Moreover, the Ketonix® presented the third highest BrAce, which would support the betahydroxybutyrate concentration in this trial. As per the remainder of the trials (aside from 'baseline' and 'twice per day training') blood glucose increased with RER and CELIF BrAce, with a decrease in bethahydroxybutyrate. Additionally, although

there were supporting findings to the BrAce findings within this trial, the overall metabolic representation was equivocal.

In summary the acetone detectors were able to present different levels of BrAce concentrations, thus suggesting that they have the ability to detect varying levels of carbohydrate availability. This was particularly highlighted within the 'baseline' and 'twice per day training' trials, where the CELIF BrAce and Ketonix® (to a lesser extent) were able to distinguish the trials that were expected to have the highest and lowest carbohydrate availability. Furthermore, the 'sleep low' trial presented a corresponding metabolic picture, where the CELIF BrAce and other metabolic measurements complemented each other. However, the remaining trials presented differing metabolic values. The variances in BrAce readings when compared to the accompanying metabolic measurements could be due to a myriad of factors, including the calibration state of the CELIF BrAce prototype, the app updates of the Ketonix® and the participant's diet the days before following the ketogenic strategy, which was not accounted for.

6.9 Incompletion of exercise protocol

In relation to the aim of this chapter, which was to explore whether the agreed protocol from Chapter 4 was practically effective with endurance athletes during exercise, the participant was unable to complete any of the 60 min 75% $\dot{V}O_{2max}$ trials. Through exploring the quantitative and qualitative data, the mechanisms that could be responsible for the participant's inability to complete the $\dot{V}O_{2max}$ trials are two-fold. Firstly, the participant's metabolic response to the nutrition strategy and exercise conditions, which highlighted their inexperience under such conditions. Secondly, the difficulty using some of the equipment could have impeded their ability to exercise. Consequently, the remainder of this section will

explore why this happened and in Chapter 7, the overall discussion will discuss these findings further and suggest relevant changes.

Despite endurance sport being determined by a myriad of factors (i.e., psychological resilience, sleep quality and physiological makeup etc.), the inability to sufficiently replenish ATP stores could be the primary reason that the participant was unable to complete the 75% $\dot{V}O_{2max}$ trials. The insufficient ability to replenish ATP stores was highlighted metabolically. For the participant to continue exercising, their muscle glucose levels needed to be replenished via glycogenolysis. However, despite an increase in capillary blood glucose concentration within three of the four "train low" strategies with an increase in RER and CELIF BrAce, and a decrease in betahydroxybutyrate concentration, the participant failed to reach 60 min. This is due to the debilitations of the glycogenolysis process. In contrast to the seemingly supportive mechanism of increase capillary blood glucose, there is an increase in concentration of its products and reliance on non-carbohydrate fuels (i.e., ketones). The products of glycogenolysis include glucose and glucose 1-phosphate, with the glucose 1-phosphate isomerizing to glucose 6-phosphate (Mul *et al.,* 2015; Jeukendrup and Gleeson, 2019; Hargreaves and Spreit, 2020; Mougios, 2020). The increase in glucose 6-phosphate concentration within the blood impedes the functioning of hexokinase, which has the function to catalyse the formation of the glucose 6-phosphate molecule (Mul *et al.,* 2015). Thus, essentially creating a case of feedback inhibition. It is suggested that this inhibition becomes important when there is an over-production in glucose 1-phosphate, and therefore glucose 6-phosphate due to the high glycogenolytic rate during demanding exercise, including the 75% $\dot{V}O_{2max}$ protocol that was implemented within this experimental pilot study (Mul *et al.,* 2015; Areta and Hopkins, 2018). Because of the increased concentrations of glucose 6-phosphate under such demanding conditions, glycogen utilisation may prevent glucose from

transporting to the working muscles to be used (Hargreaves and Spriet, 2020; Mougios, 2020). Consequently, reducing the participant's exercise capabilities. Furthermore, the participant's usual training of prolonged steady-state exercise for ultra-endurance running events did not reflect the exercise intensity used in the pilot study. The participant's lack of experience exercising at the intensity that was implemented within this study could have impeded their physiological ability to complete the exercise protocol. Furthermore, the participant expressed that they had "limited experience at exercising at intensities that were used", which suggests that the restrictions of glycogenolysis and increased reliance on ketones, would happen at an increased rate than an individual who is more accustomed to exercising at such intensities. Overall, the lack of experience exercising at the intensities used in this experimental pilot study was reflected in the participants metabolic data, which may be the main reason why the participant failed to exercise at the prescribed intensity and duration.

The Hans Rudolph 9060 mouthpiece and the accompanying nose clip and Ketonix® were the main aspects of the equipment that caused discomfort for the participant. The participant did also communicate that they became accustomed to using the Hans Rudolph mouthpiece and nose clip during exercise. However, this was not the case with the Ketonix®. Moreover, the difficulty in providing an end tidal exhale whilst exercising could have limited the practicality of the Ketonix® in this study. This was highlighted in Figure 25 where a number of the Ketonix® scores during exercise were 0 ppm, which may be because the sample the participant provided was insufficient for the Ketonix® to effectively detect and analyse the sample. Thus, the difficulty of providing a sample during exercise, could explain the Ketonix® results reported in this experimental pilot study.

CHAPTER 7 – OVERALL DISCUSSION

7.1 Introduction

The overarching purpose of this research was to establish and refine a suitable protocol, which could be employed to analyse the effectiveness of different "train low" strategies at eliciting low carbohydrate availability in endurance athletes. Six key aims were identified to guide the research. Firstly, to establish the most efficient procedure for collecting the required gas samples. Then, identify the most effective sequence to collect the measurements required. Thirdly, to compare the BrAce concentrations measured by the CELIF BrAce prototype (Chemistry Department at Durham University), and a BrAce detector currently on the market (Ketonix®). Fourthly, to understand the impact on the readings generated from adjusting the Ketonix® app settings. Fifthly, to understand whether measuring BrAce is an effective method to detect low carbohydrate availability following the implementation of "train low" strategies. Finally, to explore whether the agreed protocol for the experimental pilot study was practically effective with endurance athletes during exercise. These aims were addressed in Chapters 3-6 respectively. Chapter 7 presents an overall discussion, which discusses whether the agreed protocol for the experimental pilot study was practically effective with endurance athletes during exercise to establish a suitable protocol for future research to use. Thus, this chapter will reflect on the practicality on the protocol with suggestions to improve future practice. A synopsis of what future research should aim to do will be provided to conclude Chapter 7 after the limitations of this methodological-development study have been addressed.

7.2 Practicality of the protocol

The ordering that was decided on from the findings in Chapter 4 was effective during the data collection period with the ultra-endurance participant. However, the practicality of the Hans Rudolph mouthpiece (9060, USA) and nose-clip (9014, USA), and the 75% $\dot{V}O_{2max}$ protocol that were used within this method-focused study could be adapted moving forward to maximise the findings of future related studies. The practicality of the Ketonix® during exercise was also limiting to the protocol, however the research team did not present any developments to the Ketonix® that could be made moving forward.

7.2.1 The CELIF BrAce prototype data

Overall, the CELIF BrAce prototype produced data that generally reflected the other metabolic measures and what the literature suggested for the fasting conditions of the participants involved. This was not only partly highlighted with some of the data from the ultra-endurance athlete at rest, but also from the values the prototype presented for non-endurance athletes in Chapter 5. The prototype produced less variability in BrAce concentrations than the Ketonix®, which was particularly highlighted in Chapters 5 and 6. However, notwithstanding that the prototype produces reasonable BrAce concentrations, the number of measurements this study reported during exercise is limited due to technical faults. Faults linked to the CO_2 sensor, calibration and the programming within the machine during data collection prevented some measurements to take place, which does reduce the practicality of the prototype. Nevertheless, this prototype is in its infancy (particularly being used in a sports and exercise setting), and through the faults that the research team encountered with the prototype during data collection should support the continual development of the machine moving forward. Additionally, with the development of the

machine, the analysis coding used within the present study could also be re-evaluated to support the quality of the BrAce concentration findings in the future.

7.2.2 Mouthpieces

Originally (as discussed in Chapter 3), the impracticality of removing and reattaching the original Hans Rudolph (V2, USA) mask led to using the 9060 mouthpiece for data collection with the ultra-endurance participant. However, from communicating with the participant, they complained that the mouthpiece was uncomfortable and was hard to breath into, especially as the exercise duration increased. Also, there was a high level of saliva within the mouthpiece, which was cleaned as best as possible between each measurement, however although the RER results in Tables 14-17 appear sensible and consistent, the excess saliva may have impacted the measurements that it provided. Thus, in the future, the research team plan to use the Hans Rudolph V2 mask, which is more comfortable than the 9060 mouthpiece and leave the mask on for the duration of the trials. Then when it comes to the M3B analysis, the research team would attach the accommodating turbine, and then complete the measurements for 60 s. After the 60 s has been completed, the turbine would be removed, and the CELIF BrAce mouthpiece would be passed through the hole of mask, and the participant would then complete their CELIF BrAce measurements. The only consequence of this would be that the participant would have to hold the mouthpiece as they breathe into it, however this should not be too difficult. Then after the CELIF BrAce measurement (and if the research team decide to continue using the Ketonix®), the Ketonix® (attached to the CELIF BrAce mouthpiece) would then be passed through the V2 mask hole. In short, the protocol is as effective as it can be with the ordering of the measurements, however moving forward if the Hans Rudolph V2 facemask was swapped for the 9060 mouthpiece, taking the measurements would be more practical and comfortable for the participant.

7.2.3 Baseline testing

Despite being grounded by the literature, the participant failed to complete any of the 75% $\dot{V}O_{2max}$ trials, which impedes the validity of findings within this study. Consequently, a familiarisation protocol could be implemented during future baseline testing to account for individual differences related to their sport and physiology. This present study decided upon the 75% $\dot{V}O_{2max}$ as the intensity for the trials because 65% $\dot{V}O_{2max}$ is where the maximal rate of fat oxidation occurs, and this study aimed to understand the changes in BrAce and how effective the prototype is at detecting these changes in carbohydrate metabolism at rest and during exercise (Hawley and Morton, 2014). However, the exercise intensity was too high for the participant, which could be because they were an ultra-endurance athlete, whereby generally they are accustomed to exercising at a reduced intensity (~65% $\dot{V}O_{2max}$) for prolonged periods (sometimes for ~24 h) (Saris *et al.,* 2003; Burke, 2021). Thus, their physiological makeup would not be suited to exercising for shorter periods at a higher absolute intensity (Saris *et al.,* 2003). On the contrary, a marathon runner, who more consistently exercises at higher intensities (≥ 80% $\dot{V}O_{2max}$) may be more suited to have completed the trials (Joyner and Coyle, 2008; Tucker, 2016; Burke, 2021: Rosenburg and Sailors, 2021). Consequently, in the future, to ensure a more bespoke workload is chosen that considers the individual's sport and physiology, a familiarisation protocol could be integrated within the baseline testing. The familiarisation protocol could include the participants completing the 75% $\dot{V}O_{2max}$ protocol for as long as they can, when they are following their usual nutritional strategy (no ketogenic strategy involved). This in turn, would allow a more bespoke workload to be determined, whereby the participants' unique metabolism during exercise would be integrated to ensure they would be metabolising their carbohydrate stores, but not at a too intense or reduced intensity so that they could complete the full duration of

the trial. A familiarisation protocol would also provide another opportunity for the participant to become accustomed to the equipment.

7.2.4 Experimental protocol

After baseline testing, where the participant's 75% $\dot{V}O_{2max}$ is determined, and when the participant is ready to begin performing each of the "train low" strategies, a fuelled trial could be implemented alongside the "train low" strategies. Consequently, this would allow the research team to investigate if the CELIF BrAce detector actually detects the lower carbohydrate availability more within the "train low" strategies in comparison to the fuelled trial.

7.3 Limitations

Due to time constraints, the experimental pilot was limited to one participant. With more time, the research team could have gained a greater insight into the most effective protocol moving forward and this pilot study could have included more endurance participants. The benefit of more time would not only have included increased experience for the research team to replicate the protocol decided from Chapter 4, it would have allowed the research team to collect more quantitative and qualitative data from the participants. Additionally, the limited number of BrAce concentrations from the CELIF prototype during exercise reduces the clarity as to whether the prototype was effective in detecting the level of carbohydrate availability within the selected protocol. Consequently, the understanding for what can be changed within the prototype to attain more effective readings to help understand the metabolism of participants is limited in comparison to if the BrAce CELIF presented more data during exercise.

7.4 Future steps

Integrating what has been done within this present methodological-development study and rectifying the limitations of this study could help to distinguish a more individualised carbohydrate intake for "train low" strategies within a programme of research. The overarching aim of the subsequent programme of study is to provide more refined recommendations for "train low" strategies, whereby endurance athletes are able to commence exercise, where they are maximising their physiological adaptations to support performance, yet reducing the debilitations associated with reduced carbohydrate intake. The ketogenic strategy was used within this thesis, which is an absolute value of 50 $g \cdot d^{-1}$, and therefore does not consider individual differences between individuals (Burke, 2020). Furthermore, the next step to help understand what the individualised recommendations could be to compare different reduced carbohydrate strategies (e.g., 25 vs 50 vs 75 $g \cdot d^{-1}$) in a cross-over design and analyse the effects of different strategies on physiological adaptations and endurance performance. Moreover, to monitor the effectiveness of any adaptations, a participant could be analysed not only before and during exercise but also monitoring adaptations after exercise (Yamai *et al.,* 2009). The rationale for analysing metabolites during recovery is that this is when mitochondrial adaptations take place and when there is a natural rise in BrAce and betahydroxybutyrate (Fery and Balasse, 1983; Evans *et al.,* 2017). Thus, the research team will collect the measurements during the recovery phase (3-5 h after exercise) in addition to at rest and during exercise in the next stage of research (Fery and Balasse, 1983; Evans *et al.,* 2017). This in turn would enhance the findings in the experimental pilot study and provide a clearer insight into which reduced carbohydrate strategy elicits the most conducive adaptations for endurance athletes. Through understanding the BrAce measurements from the CELIF prototype within the experimental pilot study, the prototype prior to commencing the next stage of research can be developed by undergoing

the relevant recalibrations to create a more effective prototype for detecting BrAce levels at rest and during exercise. Finally, this experimental pilot study did not include a performance trial to assess the effectiveness of the "train low" strategies. Furthermore, to distinguish the effectiveness of following the acute carbohydrate strategies, an endurance-based performance trial will take place (e.g., a time trial) before and after a period of following a reduced carbohydrate strategy. The research team will then compare the data to learn which reduced carbohydrate strategy enhances endurance performance the most. Consequently, these results from the performance trial will support studies in finding individualised "train low" recommendations for endurance athletes.

CHAPTER 8 - CONCLUSION

The overarching purpose of this research was to establish and refine a suitable protocol, which could be employed to analyse the effectiveness of different "train low" strategies at eliciting low carbohydrate availability in endurance athletes. This method-focused thesis included four pilot studies to address the six aims of the thesis. Firstly, there was the mouthpiece and integration of sports science/chemistry equipment study, which aimed to establish the most efficient procedure for collecting the gas samples in parallel through using the LemonMedical Adaptor. However, after understanding that the LemonMedical adaptor impaired the CELIF BrAce data due to 'dead space', the research team focused on taking measurements sequentially. Thus, a specific order of measurements during exercise had to be identified, which accommodated both blood and breath/gas equipment. Through knowing that taking bloods in parallel with the Cortex M3B would practically work, due to both measurements taking a similar amount of time, this was the decided tactic to start the protocol. To further save time, having another research team member to facilitate the removal of the Hans Rudolph mouthpiece connected to the Cortex M3B supported the fluidity of taking the CELIF BrAce measurements. To improve the quality of the CELIF BrAce sample, a short period of time was required (~15 s) to allow the gas inlet to sample ambient air before the participant completed 60 s of breathing, finishing the 60 s with an end tidal breath to accentuate the removal of acetone from the breath. Again, a short period of time should be taken before finishing the sample recording, to allow all the participant's breath to be analysed and for the CO_2 levels in the prototype to return to baseline. Finally, to conclude the metabolic measurements, after the CELIF BrAce measurement was completed, a Ketonix® BrAce sample was taken.

To begin to understand the quality of the CELIF BrAce prototype and the Ketonix®, each

device was used to compare resting BrAce levels following three nutrition strategies. Overall, the CELIF BrAce produced the readings that resonated most with the additional metabolic measures and the literature, with the Ketonix® providing unpredictable scores, with a wide range of scores reported between and within participants. The wide variability of scores from the Ketonix® seemed to be caused by adjusting the settings on the Ketonix® app, with higher scores being generated when ticking the 'ketogenic box'. These findings in turn suggested that the CELIF BrAce could be more suited to be used in a subsequent study when trying to understand the metabolism of endurance athletes after following acute "train low" strategies.

Next, the research team wanted to understand whether measuring BrAce provided an indication that it is an effective method to detect low carbohydrate availability following the implementation of "train low" strategies. Again, the CELIF BrAce presented higher quality BrAce readings than the Ketonix®. This was highlighted by the CELIF BrAce distinguishing the trial with the predicted highest and lowest carbohydrate availability at rest, and generally reflecting the capillary blood glucose and betahydroxybutyrate concentrations. However, there were some strange BrAce readings from the "train low" trials that did not reflect the other metabolic measurements, which suggests that the CELIF BrAce may need to undergo some development to increase its detection of low carbohydrate availability.

Finally, the thesis aimed to explore whether the agreed protocol for the experimental pilot study was practically effective with endurance athletes during exercise. Overall, the protocol appeared satisfactory. However, the impracticality of the Hans Rudolph 9060 mouthpiece and the Ketonix® during exercise and that the participant was unable to complete the exercise trials, presented the three key aspects that needed to be re-evaluated for future study. Furthermore, to progress the research approach, integrating the Hans Rudolph V2 mask, including a familiarisation protocol and a 'normally fuelled' trial, may enhance the

practicality of the protocol to optimally analyse the effectiveness of different “train low strategies at eliciting low carbohydrate availability in endurance athletes. These adaptations could support the development of a more individualised recommendation for “train low” strategies.

CHAPTER 9 - OVERALL REFLECTION

My overall reflection is going to include considerations of my research journey during the past 10 months, specifically referring to where I started, where I finished and where this research journey is going to continue to.

I transitioned onto the research project with a excitement due to the focus of the project being around exercise metabolism. During my undergraduate degree I had not learnt about exercise metabolism specifically, however, I had gathered a basic knowledge base through the physiology and nutrition modules that I really enjoyed and was excited to learn more about the area and be challenged. In addition to the excitement, I was also apprehensive about whether I would be able to adequately adapt to the scale of the project. My anxieties were predominantly caused by the discrepancy of my motivation to deliver a helpful and meaningful research thesis and my limited starting knowledge and experience level of the chemistry-based elements and synthesising that with the sports science elements. I understood a high level of trust was placed upon me to lead such a cutting-edge project, and I did not want to waste the opportunity.

During the research project, I felt my quality as a researcher was consistently questioned, which led to the development of me as a researcher and as a person. My research project began in November 2021 and during the first six months, I felt I was not making much progress, which I think was caused by my insecurities as a researcher. Originally (until April 2022), the plan for my MRes was not to be a method-focused study, but rather to lead a cross-over randomised controlled extended version of the experimental pilot study that took place in this current thesis (Chapter 6). During this time, I was becoming accustomed to the BrAce detector and the other equipment involved, as well as broadening my knowledge of

"training low" and the subsequent metabolic adaptations that take place (e.g., mitochondria biogenesis). However, I knew that I could have been more proactive by asking more questions of my supervisors from both departments to kick start my writing and my understanding of the equipment and preparing for data collection. Even though it may be hard to understand, I was not being as proactive because I did not want to make any mistakes with the work I was doing, so I was consistently thinking about how to effectively complete my thesis optimally. However, I did not act, and instead put any concerns to the back of my mind. The turning point for my proactiveness as a researcher came in late April 2022, when I was fortunate enough to be offered a Durham Doctoral Scholarship for a three-year PhD, whereby I would be following on my research from the MRes. This was the turning point because for me to continue onto the Durham Doctoral Scholarship (which I really wanted to do), I had to complete my thesis by 30th September 2022. This meant I had a month less to complete my thesis. Although a month does not sound a great deal of time, with the amount of work I had done by April 2022, with no empirical data, I was incredibly concerned. With strong support from my supervisory team, a clear plan was created with agreed, regular deadlines to ensure I would submit before the deadline. Additionally, the focus of the thesis changed to a method-focused study, which meant I could use the information I had collected and continued to collect about the journey that were shown in the majority of this thesis. The level of accountability I felt with the task of meeting the agreed deadlines stimulated my proactiveness. I then found my increased proactiveness with reading papers, understanding the measurements, communicating with my supervisors resulted in an intensified interest in the area of exercise metabolism, which I hope has been reflected throughout this thesis. Through my increased perseverance, I gained confidence in my ability as a researcher, which my supervisors and I thought improved the quality of my work. My increased dedication also ensured I met all the agreed deadlines, which although some deadlines were tight, all the

work I needed to get done had been completed. In summary, after a slow start to my MRes journey, which was grounded by insecurity of my competencies, the personal accountability that arose from the opportunity to complete a 'follow-on' study in a PhD increased my dedication and confidence to complete my thesis.

The range of knowledge and experiences I have gained during this MRes journey has equipped me sufficiently to complete my PhD to a high standard. Firstly, the foundational knowledge I have learnt from a method and metabolic perspective has provided a solid platform to continue to develop into the PhD. I am determined to play a part of progressing the field of exercise metabolism and nutrition through my research, which I feel I can do by creating a more individualised "train low" strategy, which to my knowledge has not currently been established. In addition to furthering my knowledge, I also want to continue the development of my research experiences during the PhD. From my experiences in the MRes I have learnt the importance of regular deadlines, which are shared with the supervisory team. I will therefore ensure that I implement them within my PhD. To further my experiences in the PhD, I aim to share my research more frequently and broadly. During my MRes, I presented at one conference, which was university-based, so during the PhD, I hope to travel around Britain and Europe to various conferences to share my research and learn about other academics' research, and network with like-minded individuals who are ahead of me in my career. Through implementing my aims from a knowledge and experience perspective during my PhD, I think I will continue to develop as a researcher.

APPENDICES

Appendix 1: PAR-Q (ACSM, 2013)

Physical Activity Readiness Questionnaire (PAR-Q)

If you are between the ages of 15 and 69, the PAR-Q will tell you if you should check with your doctor before you significantly change your physical activity patterns. If you are over 69 years of age and are not used to being very active, check with your doctor. Common sense is your best guide when answering these questions. Please read carefully and answer each one honestly: check YES or NO.

1. Has your doctor ever said you have a heart condition and that you should only do physical activity recommended by a doctor? Y / N

2. Do you feel pain in your chest when you do physical activity? **Y / N**

3. In the past, have you had a chest pain when you were not doing physical activity? **Y / N**

4. Do you lose your balance because of dizziness or do you ever lose consciousness? **Y / N**

5. Do you have a bone or joint problem (for example, back, knee or hip) that could be made worse by a change in your physical activity? **Y / N**

6. Is your doctor currently prescribing medication for your blood pressure of heart condition? **Y / N**

7. Do you know of any other reason why you should not do physical activity? **Y / N**

YES to one or more questions:

You should consult with your doctor to clarify that it is safe for you to become physically active at this current time and in your current state of health.

NO to all questions:

It is reasonably safe for you to participate in physical activity, gradually building up from your current ability level. A fitness appraisal can help determine your ability levels.

I have read, understood and accurately completed this questionnaire. I confirm that I am voluntarily engaging in an acceptable level of exercise, and my participation involves a risk of injury.

Signature: Date:

Having answered YES to one of the above, I have sought medical advice and my GP has agreed that I may exercise.

Signature: Date:

Note: This physical activity clearance is valid for a maximum of 12 months from the date it is completed and becomes invalid if your condition changes so that you would answer YES to any of the 7 questions.

Appendix 2: Energy availability questionnaire

Adapted from: Keay, N., Francis, G. and Hind, K. (2018). Low energy availability assessed by a sport-specific questionnaire and clinical interview indicative of bone health, endocrine profile and cycling performance in competitive male cyclists. *BMJ Open sport & Exercise Medicine*, 4(1), pp 1-7.

Study of endurance athletes

Participant information:

Name:

Age:

DOB:

Height (m):

Weight (kg):

Contact:

Email:

Mobile number:

Address:

Endurance exercise history:

Current endurance racing distance:

Years of endurance training:

Training load: average hours of endurance exercise per week

Training load: any strength and conditioning or other exercise?

Previous sports to endurance exercise:

Nutrition

Are you weight steady? (Do you maintain a consistent weight)

Vegetarian/food intolerances?

Do you take any supplements?

Number of fasted rides per week:

Fuelling during ride greater than 1 hour:

Post ride fuelling:

Average number of portions dairy per day:

Average caffeine per day (coffee/gels etc):

Medical

Past Medical History:

Injury history:

Drug History (current and previous, including any courses steroids):

Current or past smoking?

Weekly alcohol units?

Any common medical issues within your family that we should be made aware of?

Appendix 3: Semi-structured interview questions for MRes Study

How did you find the testing experience?

How did you find following the ketogenic diet? Psychologically and physically?

How did you find exercising after following the ketogenic diet? Psychologically and physically?

Would you look to implement “training low” into your future programmes?

Were things explained clearly to you during the testing process? Was there anything you found difficult to understand?

How comfortable was the equipment to use? Any issues - why?

Any other thoughts?

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