ENDOGENOUS GLUCOSE PRODUCTION AND GLUCONEOGENESIS DURING EXERCISE IN ATHLETES ON EITHER A LOW-CARBOHYDRATE OR MIXED DIET

by

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DECLARATION

I, Christopher Webster, know the meaning of plagiarism and declare that all of the work in the dissertation/thesis, save for that which is properly acknowledged, is my own.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
ASA24	Automated Self-Administered 24-hour Recall
ATP	Adenosine triphosphate
βHB	beta-hydroxybutyrate
BMI	body mass index
CHO	carbohydrate
EGP	endogenous glucose production
ESSM	Exercise Science and Sports Medicine
FA	fatty acids
FFA	free fatty acids
GCMS	gas chromatography–mass spectrometry
GLY	liver glycogenolysis
GNG	gluconeogenesis
HOMA-IR	reduced homeostatic model assessment of insulin resistance
HR	heart rate
HR_{max}	maximum heart rate
IMTG	Intramuscular triglyceride/s
IR	insulin resistance
LCHF	low-carbohydrate high-fat
MPE	molar percent enrichment
MUFA	monounsaturated fatty acids
OGTT	Oral glucose tolerance test
PPO	peak power output
PUFA	polyunsaturated fatty acids
Ra	rate of appearance
RER	respiratory exchange ratio
Rd	rate of disappearance
RPE	rating of perceived exertion

TG	triglyceride/s
UCT	The University of Cape Town
VCO_2	CO_2 expiration
VE	ventilation volume
VLDL	very low density lipoprotein/s
VO_2	oxygen consumption
VO_{2max}	maximum oxygen consumption

ABSTRACT

INTRODUCTION. The LCHF diet produces major changes in whole-body substrate metabolism and energy stores such as reduced muscle and liver glycogen content, increased rates of fat oxidation and decreased rates of carbohydrate (CHO) oxidation. Despite reduced CHO availability, the rate of CHO oxidation that can be sustained during exercise in LCHF athletes is surprisingly high. The most probable source of this glucose is via the process of gluconeogenesis (GNG). However, endogenous glucose production (EGP) and GNG has not been studied during exercise in athletes on a LCHF diet. Therefore, the aim of this study was to determine if there are differences in EGP, GNG and glycogenolysis (GLY) during exercise in endurance-trained athletes who habitually eat either a mixed or LCHF diet.

METHODS. Fourteen (7 LCHF, 7 Mix) endurance-trained male cyclists (VO_{2max} 61 ± 5 ml/kg/min LCHF; 63 ± 8 ml/kg/min Mix), matched for age (36 ± 6 y LCHF; 32 ± 5 y Mix), body composition (BMI 23.6 ± 1.8 LCHF; 23.4 ± 2.0 Mix) and relative peak power output (4.8 ± 0.4 W/kg LCHF; 5.0 ± 0.4 W/kg Mix), were recruited. Diets were analysed using the Automated Self-Administered 24-hour Recall (ASA24) analysis software. Participants cycled for 2 h at 55% of peak power output during which EGP was measured by infusion of [6,6-$^2H_2$]glucose, and fractional gluconeogenesis was measured by ingestion of ^2H_2O. Blood samples were collected at regular intervals for isotope enrichment analysis.

RESULTS. Rates of GNG were similar during exercise in both the LCHF and mixed diet groups (2.8 ± 0.4 mg/kg/min LCHF; 2.5 ± 0.3 mg/kg/min Mix). The rates of GLY during exercise were significantly higher in the mixed diet group than the LCHF group (3.2 ± 0.7 mg/kg/min LCHF; 5.3 ± 0.9 mg/kg/min Mix) which resulted in significantly higher rates of EGP in the mixed diet group (6.0 ± 0.9 mg/kg/min LCHF; 7.8 ± 1.1 mg/kg/min Mix). There were significant differences in the mean fat oxidation rates (1.2 ± 0.2 g/min LCHF; 0.5 ± 0.2 g/min Mix) and CHO oxidation rates (1.3 ± 0.5 g/min LCHF; 3.1 ± 0.5 g/min Mix). Blood beta-hydroxbutyrate (βHB) concentrations were significantly higher in the LCHF group than in the mixed diet group throughout exercise but there were no differences in plasma glucose, plasma lactate, serum insulin or serum FFA concentrations. The diets of the two groups differed only in fat and CHO intake (%Protein / %Fat / %CHO: 21/72/7 LCHF; 16/33/51 Mix).

DISCUSSION. Rates of fat oxidation and CHO oxidation were not associated with the rates of GNG. Apart from βHB, the precursor, substrate and insulin concentrations were remarkably similar in both groups and may have influenced GNG similarly in both groups. We conclude that rates of GNG are relatively stable across a broad range of habitual diets that can significantly alter substrate utilisation, and that dietary CHO modulates the rates of EGP via alterations in rates of GLY, both at rest and during exercise.

1) INTRODUCTION

Many South African endurance athletes follow a low-carbohydrate high-fat (LCHF) diet for reasons related to general health [44], management of specific metabolic conditions [48, 120], body composition management [16, 35, 118] and endurance performance. While the effect of this diet on exercise performance is still uncertain [114], the diet does produce major changes in whole-body substrate metabolism and energy stores. Some of these changes include reduced muscle and liver glycogen content [10, 113], increased rates of fat oxidation and decreased rates of carbohydrate (CHO) oxidation [53, 126]. Of interest is that despite a decreased reliance on CHO for energy production, the rate of CHO oxidation that can be sustained during exercise in LCHF athletes is surprisingly high [93, 163]. Since liver and muscle glycogen stores are low after a LCHF diet, the most probable source of this glucose is via the process of gluconeogenesis (GNG). However, endogenous glucose production (EGP) and GNG has not been studied in athletes on a LCHF diet and it is unclear whether these athletes do have higher rates of GNG than athletes who consume a diet higher in CHO. Therefore, this review will discuss literature on substrate metabolism during exercise in both high-CHO and LCHF athletes, with a particular focus on endogenous glucose production and the regulation of GNG.

1.1) Overview of substrate metabolism. A basic overview of substrate metabolism is necessary to understand the interaction between fat and CHO metabolism and how dietary manipulation can influence substrate utilisation. CHO, in the form of glucose, and fat, in the form of fatty acids (FA), are the main substrates oxidised for energy by the muscles both during rest and exercise [134, 156]. In general, fat stores are abundant in the body but take longer to mobilise, while CHO stores are limited in quantity but can be rapidly converted to energy [63]. These substrates are stored both close to the site of oxidation within the muscle and elsewhere in the body where they can be released into the circulation. The storage form of CHO is a string of glucose molecules known as glycogen while fat is stored as three fatty acid chains bound by a glycerol molecule to form triglycerides (TG). Muscle glycogen and intra-muscular triglycerides (IMTG) are readily available substrate stores for energy production due to their close proximity to the working muscle but are limited in energy content [154].

Therefore, during prolonged activity, the active muscles also rely on glucose and FA to be imported via the circulation from other sources. The liver stores glycogen which is broken down to glucose in the process of glycogenolysis (GLY) and is released into the circulation, whereas TG are stored in

adipose tissue throughout the body and are released into the circulation as free fatty acids (FFA). Additionally, the liver is capable of manufacturing both glucose and TG. Glucose can be produced from a variety of precursors, mostly in the liver but also to a lesser extent in the kidneys [108], via the process of gluconeogenesis (GNG). Precursors for GNG include most amino acids (primarily alanine), lactate and glycerol [136]. Glucose produced via GNG and liver GLY together constitute endogenous glucose production (EGP). FA can be manufactured by the liver from CHO via the process of de novo lipogenesis and TG formed in the liver is released into the circulation within very low density lipoproteins (VLDL).

Lastly, fat and CHO can be acquired exogenously via the ingestion of fat- and CHO-containing foods. Ingested glucose is absorbed rapidly into the circulation where it is immediately available for uptake by active tissues. Fats take longer to digest and absorb and are re-esterified into TG before being transported to the circulation via lymphatic lacteals within lipoprotein particles called chylomicrons. In order to be transported to tissues such as muscle, triglycerides within the VLDL and chylomicrons are broken down into FA within the capillaries by the enzyme lipoprotein lipase. These FA are then transported into the muscle and mitochondria for beta-oxidation [81].

1.2) Substrate metabolism during endurance exercise. The energy needs of the muscles are therefore met by contributions from muscle glycogen and IMTG stores as well as a steady supply of circulating glucose, FFA and lactate [45], which must be carefully maintained to ensure sufficient substrate reaches the exercising muscle and other active tissues. The relative contribution of these fuels towards muscular energy production is dependent on a number of factors, such as exercise intensity, exercise duration, hormonal milieu, plasma substrate concentrations and nutritional strategies. Each of these factors will be discussed in more detail in the following sections with particular attention to their role in plasma glucose homeostasis.

1.3) Exercise intensity. Energy expenditure increases in proportion to exercise intensity. At rest and during exercise at very low intensities (± 25% VO_{2max}), the demand for energy production is low and plasma FFA contributes approximately 80% of energy production, with plasma glucose contributing most of the remaining 20% [134, 156]. There is a 6- to 10-fold increase in the rate of energy expenditure from rest to exercise at moderate intensities (50% - 75% VO_{2max}) [134, 156]. To meet this higher demand for energy production, substrate mobilisation and utilisation is increased. In athletes living on high-CHO diets, the rate of plasma FFA oxidation peaks at these moderate

intensities and there is increased muscle glycogen and IMTG utilisation, which together contribute about 50% of energy production. At high exercise intensities (≥ 75% VO_{2max}), the need for rapid energy production favours the utilisation of CHO as it can rapidly produce ATP in the cytosol via the oxygen independent glycolytic pathways [63]. Muscle glycogen becomes the dominant fuel source, contributing over 60% of energy production, and plasma glucose is the other major source of CHO [134, 156]. Conversely, the contributions of plasma FFA and IMTG to energy production decreases and eventually becomes negligible at exercise intensities above 85% of VO_{2max} [3].

The contribution of plasma glucose to energy production increases in proportion to the exercise intensity [134, 156]. Therefore, in order to maintain euglycaemia, the rate of EGP increases to match plasma glucose utilisation. The rate of EGP doubles from rest to exercise at approximately 50% of VO_{2max} [152] and can increase up to 4-fold from rest to exercise at 75% VO_{2max} [46]. The rate of both GLY and GNG increases to produce this increase in EGP, but Trimmer et al. [152] and Emhoff et al. [46] have shown that GLY increases to a greater degree than GNG and is responsible for as much as 80% of EGP during exercise. The importance of GLY to EGP increases as exercise intensity increases [90]. However, glucose produced via GNG can be derived from a number of different precursors. In the case of Trimmer et al. [152], only GNG from glycerol was measured, whereas Emhoff et al. [46] measured GNG from lactate. This means that a portion of GNG was potentially not reported and the true contribution of GNG to EGP may have been underestimated and the contribution of GLY overestimated [4]. Total GNG from all precursors has not been well researched during exercise in trained athletes.

1.4) Exercise duration. As a bout of endurance exercise progresses, there are changes in substrate utilisation as the limited CHO stores in the muscle and liver are gradually depleted. Usually glycogen content is high at the start of exercise, resulting in a high rate of EGP and increased CHO oxidation early in exercise [8]. As glycogen stores diminish, high rates of EGP and energy production from CHO cannot be sustained and there is a gradual shift towards increased fat oxidation [6]. Liver GLY is particularly important in the maintenance of euglycaemia [46, 152]. Blood glucose concentrations are usually well maintained at the start of exercise but gradually decline as EGP is no longer sufficient to sustain euglycaemia [32]. This is particularly true during exercise after an overnight fast, which can reduce liver glycogen content by around 40% [113]. Plasma glucose concentrations are better maintained after a recent CHO-containing meal or snack [38]. However, without the ingestion of CHO, plasma glucose concentrations will eventually decline to around 2.5 - 3 $mmol \cdot L^{-1}$ which is associated with the termination of exercise and symptoms of hypoglycaemia [32, 49]. During

prolonged low intensity exercise, glucose from GNG and FFA oxidation may be sufficient to maintain exercise [90].

1.5) Hormonal milieu. There is a strong hormonal regulation of plasma glucose concentration which impacts on both CHO and fat metabolism. Plasma glucose concentration, which is optimally maintained between 4 and 6.5 $mmol \cdot L^{-1}$, is a balance between glucose uptake from the circulation by active tissues and glucose entry into the circulation from endogenous and exogenous sources. When there is an increase in the plasma glucose concentration, usually as a result of exogenous CHO from the diet, insulin is released into the circulation by the beta cells of the pancreas. Insulin inhibits EGP but promotes the uptake and oxidation of glucose in the muscle, the storage of glucose as glycogen in the liver and muscle and the production and storage of triglycerides in adipose tissue. On the other hand, low plasma glucose concentrations produce the combination of low insulin and increased glucagon, catecholamine and glucocorticoid concentrations that promote the mobilisation of energy substrates. Under these hormonal conditions there is increased lipolysis (the breakdown of TG) and EGP and GNG are stimulated. This feedback regulation ensures that a normal blood glucose concentration is maintained.

1.6) Plasma substrate concentrations. In addition to hormonal regulation, the liver is able to respond directly to plasma substrate concentrations [109]. One of the main substrates that exerts direct control over EGP is glucose. Glucose delivery to the liver influences the rate of EGP such that hyperglycaemia can inhibit glucose production while hypoglycaemia has a stimulatory effect on glucose output [99, 109, 141, 148]. This mechanism plays an important role in the matching of EGP to glucose uptake from the circulation [149]. Another substrate which has a direct regulatory effect on EGP is FFA. Elevated FFA concentrations usually occur during periods of fasting [137] where CHO availability is low. Under these conditions, an increase in plasma FFA concentration may directly stimulate the production of glucose via GNG [13, 26, 30, 54]. Plasma ketone concentrations are related to high rates of fat oxidation and may also play an important role in the regulation of GNG. Ketones suppress GNG from amino-acid precursors, thereby protecting protein stores [140]. They are also a precursor for GNG [52], but the role of ketones in the regulation of GNG has not been well researched.

Gluconeogenic substrate delivery to the liver is another factor which can influence the rate of GNG [56, 107]. Lactate is considered the primary precursor for hepatic GNG [6, 34, 106] and is especially

important during exercise, when plasma lactate concentrations increase in proportion to exercise intensity [134]. However, glycerol becomes an important precursor during prolonged fasting [78, 95] or during prolonged exercise when plasma FFA concentration and rates of lipolysis are increased [6]. During high intensity exercise, there is a marked reduction in hepatic blood flow [160] which results in reduced precursor delivery to the liver and potentially limits GNG [147]. Therefore, at high exercise intensities, there is abundant lactate availability but delivery to the liver may limit GNG; while at low/moderate exercise intensities, the lower availability of lactate as a precursor may limit GNG [46].

An increase in GNG does not necessarily result in an increase in EGP. Hepatic autoregulation is a process by which EGP is matched to glucose uptake from the circulation, such that increases in GNG are matched by equivalent reductions in GLY [79, 132, 146, 150] and vice versa [143]. Increased precursor availability may also selectively inhibit GNG from other precursors. In other words, the increase in GNG that is facilitated by an increase in precursor availability is due to an increase in glucose production from that particular precursor, while GNG from other precursors is inhibited [75, 145]. In this way, total EGP remains unchanged despite increases in GNG. Unchanged EGP despite increases in GNG has also been reported during exercise [46, 151].

Since glucoregulatory hormones are influenced by, and also exert profound effects on plasma substrate concentrations; the regulation of EGP and GNG via hormonal factors, substrate concentrations and precursor availability are intricately linked [5, 27].

Liver glycogen content is an additional factor which regulates the rate of EGP. In particular, the CHO content of the diet is positively associated with EGP and CHO oxidation rates in the post-absorptive state [12, 139]. This effect was primarily modulated via changes in GLY and not in GNG [12]. When the macronutrient composition of the diet was altered to the extremes of CHO and fat intake (85% fat, 15% protein versus 85% CHO and 15% protein), there were only minor differences in GNG despite very large changes in GLY [12]. The rate of GNG therefore appears relatively stable regardless of dietary intake while the rate of GLY is determined by the CHO content of the diet [12, 116]. Very high-CHO intakes even increase the rate of EGP in excess of CHO oxidation needs, thereby disrupting hepatic autoregulation [12, 31, 139]. In this way, excessive GLY due to a very high-CHO diet may play a role in the development of poor glucose control [12].

1.7) High CHO nutritional strategies. Clearly, optimal substrate availability is very important for exercise performance and exercise scientists have investigated numerous nutritional strategies to

optimise substrate storage prior to exercise and its availability during exercise. In one of the earliest such studies, Christensen and Hanson in 1939 [29] demonstrated that a high-CHO diet enhanced endurance performance, increased the amount of carbohydrate oxidised for energy and maintained normal blood glucose concentration for longer. Conversely, they showed that a low-carbohydrate diet increased the amount of fat oxidised, resulted in a rapid fall in blood glucose concentration and reduced endurance performance. The benefits of a high-CHO diet were further reinforced with the development of the needle muscle biopsy technique in the 1960s. It was found that the CHO content of the diet was positively associated with muscle glycogen content [10]. Furthermore, a very strong association was found between initial muscle glycogen content, CHO-oxidation rates and time-to-exhaustion in endurance exercise [10, 69]. This evidence suggested that CHO is the optimal fuel for endurance exercise and that maximising CHO stores and oxidation is important for exercise performance.

Additionally, the supplementation of CHO during exercise may improve exercise performance [80], especially after an overnight fast [39, 40]. Ingesting CHO facilitates the maintenance of high CHO-oxidation rates during exercise after a high-CHO diet [39] and lowers the rate of EGP required to maintain euglycaemia [85]. This is known as a liver glycogen 'sparing' effect. Surprisingly, exogenous CHO did not significantly alter the rate at which muscle glycogen was used during exercise and therefore did not lead to a 'sparing' of muscle glycogen [36, 39].

Based on these and numerous other studies, athletes have been advised to maximise muscle glycogen stores by CHO-loading with a high-CHO diet for several days before an event [38]. A high-CHO pre-exercise meal and/or snack was recommended to top-up liver glycogen content [38], which in conjunction with CHO ingestion at as high a rate as could be tolerated [80], would spare liver glycogen and maintain an optimal plasma glucose concentration. A high-carbohydrate meal shortly after exercise was recommended to facilitate glycogen re-synthesis to pre-exercise levels and enable repeated exercise bouts in quick succession [38]. These recommendations for endurance exercise have become standard practice by many endurance athletes today and thus, a 75kg well-trained endurance athlete might eat in the region of 500 - 600g of CHO per day and an additional 90g/h during exercise [80].

1.8) LCHF nutritional strategies. During this period when researchers were focused on maximising CHO stores and oxidation rates, little interest was placed on fat as a fuel for exercise. This was despite the fact that many of the metabolic and physiological adaptations to endurance training relate to improvements in fat utilisation, which suggests that strategies to optimise fat metabolism

might provide an advantage in endurance exercise [65, 70]. Some of these training adaptations include: larger and more numerous mitochondria [70]; increased capillary density of muscle [7]; increased IMTG stores [110, 155]; and an increase in beta-oxidation enzymes [84]. These adaptations effectively increase the oxidative capacity of muscle and are associated with greater utilisation of fat after training at both the same absolute and relative exercise intensities [2, 68, 88]. A limitation of a high-CHO intake is that it creates a heavy reliance on glycogen and exogenous CHO in order to maintain high rates of CHO oxidation during exercise. Athletes adapted to high-CHO diets are at risk of reaching a point at which glycogen stores are no longer sufficient to meet the glucose requirements of both the central nervous system as well as the active muscles. This phenomenon, known as "hitting the wall", is distinctly different from general fatigue and is often accompanied by symptoms of hypoglycaemia [17]. The athlete is then forced to reduce exercise intensity until blood glucose concentration rises and the symptoms of hypoglycaemia have been reversed [32, 82].

It was in this very CHO-centric environment that Phinney et al. [126] in 1983 studied exercise performance in five elite cyclists who adapted to a low-CHO ketogenic diet over a period of 28 days. Unlike previous studies in which CHO restriction resulted in decreased performance [10, 29, 51, 128], Phinney et al. [126] concluded that cyclists were able to maintain endurance performance after four weeks of eating a diet which contained only 20g of CHO per day. The authors presented the concept of 'adaptation' to CHO-restriction, in which the fat-adapted athlete is able to maintain performance by oxidising fat at a sufficiently high rate to compensate for the lower CHO availability. The maximum rate of fat oxidation typically reported in athletes eating CHO-rich diets is in the region of 0.4 to 0.7 g/min [1, 157], whereas rates of 1.2 to 1.5 g/min have now been measured in elite athletes after fat-adaptation [126, 135].

There are three important differences between the Phinney study and the earlier studies which showed detrimental effects of LCHF diets on performance. The first was related to the diet duration. Participants in the Phinney study were given 28 days to adapt to the LCHF diet [126], compared to 3 – 14 days in the previous studies [10, 29, 51, 128]. The longer time period may have been important for a complete shift in metabolism to occur and for the athletes to adjust to this new metabolic state. A second key difference was the intention of the researchers. Phinney et al. [126] set out to investigate the potential for athletes to adapt to a state in which fat is their primary fuel. The athletes were carefully monitored and provided with mineral supplements when required, in order to assist with the transition into a CHO-restricted state. By contrast, the aim of the LCHF diet in previous studies was to limit glycogen re-synthesis and study the role of glycogen, especially at low concentrations, on exercise performance [10, 128]. There was no thought of facilitating an

adaptation towards fat metabolism since the exclusive focus was on CHO, especially glycogen metabolism [10, 33, 51, 128].

The third important difference was the use of a low-CHO ketogenic diet which was modelled on the diet of Arctic explorer Vilhjalhar Stefansson, who remained in good health for one year despite eating almost no CHO [98, 103, 104]. In fact, the LCHF diet in the Phinney study was so low in CHO that the plasma concentration of ketones (or ketone bodies) was significantly elevated at rest and during exercise [126]. Ketones are an energy substrate produced in the liver under conditions of very low CHO intake as a result of increased fatty acid availability [105]. They are important in that they can be oxidised by the CNS, which would otherwise be dependent solely on glucose for energy [21, 22, 117]. After adaptation to the ketogenic diet, the athletes in Phinney's study were in a state of nutritional ketosis, in which the blood ketone concentrations were elevated to a safe physiological range between 0.5 and 3 $mmol \cdot L^{-1}$ [126]. In addition to the CNS, skeletal and cardiac muscle may also effectively oxidise ketones [131] but whether ketones make a significant contribution to energy production in skeletal muscle and contribute towards the 'sparing' of muscle glycogen is not clear [37]. There is also growing evidence that ketogenic diets are an effective treatment for a number of medical conditions such as epilepsy and obesity [120]. Phinney and colleagues [126] believed that ketosis was a key feature of the successful adaptation of their athletes to a CHO-restricted diet. In theory, once in a fat-adapted ketogenic state, the muscles will have a steady and abundant supply of FFA from adipose tissue and the CNS will have a steady and stable supply of ketones and glucose from GNG.

Since the study by Phinney et al. [126] in 1983, there have been many investigations into the performance-enhancing potential of 'fat-adaptation'. Generally, fat-adaptation refers to eating a LCHF diet for long enough to shift whole-body metabolism away from glucose and towards fat as a fuel, although it is interesting to note that the ketogenic diet used by Phinney et al. [126] has not been replicated in any subsequent LCHF study [125]. Rather, many researchers have employed diets higher in CHO [53, 65, 67, 93, 135, 158] and/or tried to combine the benefits of fat-adaptation with the benefits of high-CHO intake using a strategy known as 'fat-adaptation CHO-restoration' [18, 19, 24, 60, 92, 135]. Some of these studies suggest that fat-adaptation may result in a performance advantage [92, 93], some suggest fat-adaptation may result in a disadvantage [60, 65], but the majority show no difference in time-to-exhaustion [67, 93] or time-trial performance [18, 19, 24, 53, 60, 135, 158] between LCHF athletes and a control diet higher in CHO.

1.9) *Study rationale.* It is clear from the studies mentioned above that a LCHF diet is not categorically inferior to a high-CHO diet from an endurance performance perspective. However, athletes on LCHF diets have very different metabolic profiles to athletes on diets higher in CHO. Many of the differences occur in areas which could reasonably be expected to influence EGP and in particular GNG. For example, after adaptation to a LCHF diet, CHO oxidation rates are low during exercise [93, 126] and therefore the rate of EGP is probably also low. Compared to athletes on high-CHO diets, LCHF athletes have higher rates of fat oxidation [53, 93, 126], and in some cases higher plasma FFA concentrations have been reported [128, 163]. This could result in a stimulatory effect on GNG. Plasma GNG precursor concentrations may also differ during exercise in LCHF compared to high-CHO athletes. Increased plasma glycerol concentrations [53], associated with increased utilisation of IMTG [86], could potentially increase the plasma precursor supply while reduced muscle glycogen oxidation may alter lactate dynamics [57, 96] and the availability of plasma lactate during exercise. Additionally, muscle glycogen content is reduced after a LCHF diet [93, 126] and the contribution of GLY to EGP is therefore likely to be very low.

Although GNG is relatively stable across a wide range of dietary intakes at rest [116], the combined effect of all the diet related factors mentioned above on GNG during exercise is unknown. An interesting observation is that sustained CHO oxidation rates between 1.0 and 1.5 g/min have been reported in fat-adapted athletes during endurance exercise in the fasted state [93, 163]. These CHO oxidation rates are lower than in participants eating higher-CHO control diets (2.0 to 2.5 g/min), but in the context of very limited dietary CHO and reduced muscle and liver glycogen availability, it is unclear from where this CHO was derived. It is therefore tempting to hypothesise that athletes on a long-term LCHF diet may have a heavy reliance on GNG for their CHO needs [93, 128, 159] compared to those on higher CHO diets. However, the rates of EGP as well as the relative contributions of GNG and GLY in LCHF athletes have yet to be studied during exercise and were investigated in the current thesis.

1.10) Aims and objectives. The aim of this study was to determine if there are differences in EGP, GLY and GNG during exercise in endurance-trained athletes who habitually eat either a high-CHO or LCHF diet. Our primary objective was therefore to use stable isotope tracers to measure EGP and the total fractional contribution of GNG to EGP in seven healthy male cyclists who had habitually eaten a diet containing less than 50 g CHO per day for at least the previous six months. This group was compared to seven healthy 'control' cyclists matched for age, cycling ability, weight and BMI that had habitually eaten a high-CHO diet containing over 400 g CHO per day for at least the previous six months.

2) METHODOLOGY

2.1) Ethical considerations: This study was approved by the University of Cape Town (UCT), Faculty of Health Sciences, Human Research Ethics Committee (REF: 453/2012, first approval 13-Nov-2012, final version approval 07-Feb-2014). All participants were informed of the nature of the study and written informed consent was obtained before participants were enrolled in the study.

2.2) Participants and inclusion/exclusion criteria. Fourteen endurance-trained male cyclists participated in this investigation. Participants were eligible if they were: free from known metabolic conditions; not currently taking any medications; able to answer 'no' to all the questions on a physical activity readiness questionnaire (Appendix 1); between the ages of 18 and 55 years; weight stable for a minimum of six weeks (2 kg either side of current weight); diet stable for a minimum of six months; habitually eating a self-selected LCHF or high-CHO diet; a well-trained male cyclist with at least two years of cycling experience and were actively competing and/or training for at least the past three months. An additional consideration was that the high-CHO group were matched for age, weight and cycling ability to LCHF group. Participants in the LCHF group were required to habitually eat less than 50 g of CHO per day or no more than 10% of total calories as CHO. Participants in the LFHC group were required to habitually eat over 400 g of CHO per day or 60% of total calories as CHO.

2.3) Overview of study design. The participants were required to visit the Exercise Science and Sports Medicine (ESSM) laboratories at UCT on four occasions. The first visit consisted of anthropometric measurements, capturing of the three-day diet logbook into ASA24 for screening purposes and a peak power output (PPO) test for the determination of PPO and VO_{2max}. Eligible participants then performed two exercise trials which were separated by three days. The first was a familiarization 2 h ride at 55% of PPO and the second was the tracer infusion trial, during which EGP and fractional GNG were measured. An oral glucose tolerance test (OGTT) was performed at the fourth visit which took place at least three days after the tracer infusion trial.

2.4) Recruitment and screening. Recruited participants were respondents to a press release or advertisements sent out to local cycling clubs and cycling forums. Preliminary health, weight, diet

and exercise eligibility criteria were assessed with the use of detailed questionnaires (Appendix 2). A non-quantitative food frequency questionnaire which contained foods both high in CHO and high in fat was administered as an initial dietary screening tool (Appendix 3). Potential participants were then asked to keep a three-day diet logbook, which included at least one exercising day, to get a detailed analysis of their diets. Participants were counselled on how to accurately record their food intake. Foods/dietary information from the logbook was captured by the investigator with the assistance of the participant into the Automated Self-Administered 24-hour Recall (ASA24) analysis software. This programme is based on the USDA Automated Multiple-Pass Method which has been previously validated [89, 111]. Diet eligibility was evaluated based on this ASA24 quantification of the diet. This initial ASA24 screening also served as a familiarisation of the diet logbook and data capture which was done for three days before the tracer infusion trial.

2.5) Diet. Participants who qualified for the trial were asked to maintain their habitual diet for the duration of the study. In the three days prior to the tracer infusion trial, a second detailed diet logbook was kept by the participant. This logbook was captured into ASA24 on the day of the tracer infusion trial as previously described. All the reported dietary data was from this second analysis.

2.6) Body composition. Body fat percentage was determined from skinfold measurements using the Jackson and Pollock equations [73]. Skinfold thickness (mm) was recorded at the following sites: chest; abdominal; thigh; tricep; subscapular; suprailiac; and midaxillary and body fat (%) was calculated according to the equations in section 2.16.

2.7) Peak power output. The PPO test and all subsequent submaximal exercise trials were performed using the participants' own bicycles mounted on a cycle ergometer (Computrainer Pro 3D, RacerMate, Seattle, Washington, USA), which was calibrated in accordance with Davison et al [42]. After a 10 min self-paced warm-up and 5 - 10 min recovery, the PPO test was started at a work rate of 100 W and the load increased incrementally by 20 W each minute until the cyclist could no longer sustain a cadence greater than 70 rpm or was unable to continue with the test. All participants were verbally encouraged to continue cycling for as long as possible. During the PPO, as well as during the subsequent sub-maximal trials, ventilation volume (VE), oxygen uptake (VO_2) and carbon dioxide production (VCO_2) were measured using a breath-by-breath gas analyser (Jaeger Oxycon Pro, Hoechberg, Germany) and averaged over 15 second intervals [101]. Criteria for VO_{2max}

were that two of the following three conditions were met: an end HR within 10 beats of age predicted max (200 – age); an end RPE of 19 or higher on the Borg 6-20 RPE scale [14]; and an end RER of at least 1.05 in the LCHF group and at least 1.15 in the mixed group. The average workload over the last minute of the test was taken as the PPO and the highest 15-second VO_2 value was taken as VO_{2max}. Maximal heart rate (HR_{max}) was taken as the highest HR reached during the PPO test. The Oxycon Pro, which has been previously validated against the Douglas Bag system [130], was calibrated using CO_2 and O_2 of known concentration and a cylinder of known volume, immediately before each trial. Heart rate (HR) was measured continuously during all trials using a Suunto T6 heart rate monitor (Suunto Oy, Vantaa, Finland) and HR data were recorded over 2-second intervals.

2.8) Exercise protocol. Participants performed two laboratory rides separated by exactly three days. The first served as a familiarisation ride and to standardise the participants' exercise prior to the tracer infusion trial. Participants were allowed a light/recovery session the day after the familiarisation and no exercise was permitted the day prior to the tracer infusion trial. The exercise protocol consisted of a 2 h ride, which started at a power output equal to 100 W less than 55% of PPO. The power was continuously increased up to 55% of their PPO over the first 12 min to allow the participants to warm up and then remained at this intensity for the remainder of the ride.

2.9) Stable isotope protocol and tracer infusion trial. The stable isotope protocol was based on Chacko et al. [25] modified for exercise in accordance with Trimmer et al. [152]. A blood sample for baseline ^2H_2O enrichment was obtained the day before the trial, prior to the ingestion of ^2H_2O. Participants ate an evening meal of their choice, but were instructed to keep it in accordance with their habitual diets. This meal occurred between 18h30 and 19h00, after which they fasted until the end of the trial. Participants ingested 4 g/kg body weight deuterium oxide [^2H_2O, 99% atom % ^2H] between 21h00 and 23h00 on the night before the trial to enrich body water to approximately 0.5% ^2H_2O. On the morning of the tracer infusion trial, participants arrived at the laboratory at 06h00. They lay down on a plinth and remained supine until the start of exercise. A cannula was inserted into an antecubital vein of each arm. The right cannula was used to sample blood and the left was used for the infusion of [6,6-2H2] glucose. A heating blanket was placed over the lower right arm to ensure that the blood sampled was arterialized. At 06h30 a 36 µmol/kg (6.5 mg/kg) [6,6-$^2H_2$] glucose prime was injected, followed by the start of a continuous infusion of [6,6-$^2H_2$] glucose at a rate of 0.3

µmol/kg/min (0.054 mg/kg/min) (Travenol Auto Syringe Model 5C, Travenol Laboratories Inc, Hooksett, NH, USA). Blood samples were obtained prior to the start of the [6,6-$^2H_2$] glucose infusion (-120 min) and at -20, -10 and 0 min for the determination of resting EGP and fractional GNG. A muscle biopsy was performed on the right leg (see section 2.13 for details) at approximately 08h15 (~ -15 min) and the participant was moved to the bicycle just before the start of exercise (~ -3 min). After two hours of infusion, exercise began at ~08h30 and the [6,6-$^2H_2$] glucose infusion rate was doubled to 0.6 µmol/kg/min (0.108 mg/kg). At the end of the 2 h exercise trial, the participant recovered in a supine position for 30 min. Blood samples were obtained at 90, 105 and 120 min for the determination of exercise EGP and fractional GNG. Samples for plasma glucose and lactate, serum FFA and insulin and whole blood βHB concentrations were obtained at 0, 30, 60, 90, 120 and +30 min. A post-exercise muscle biopsy was taken on the left leg immediately after exercise (see section 2.13). An overview of the protocol is presented in Figure 1.

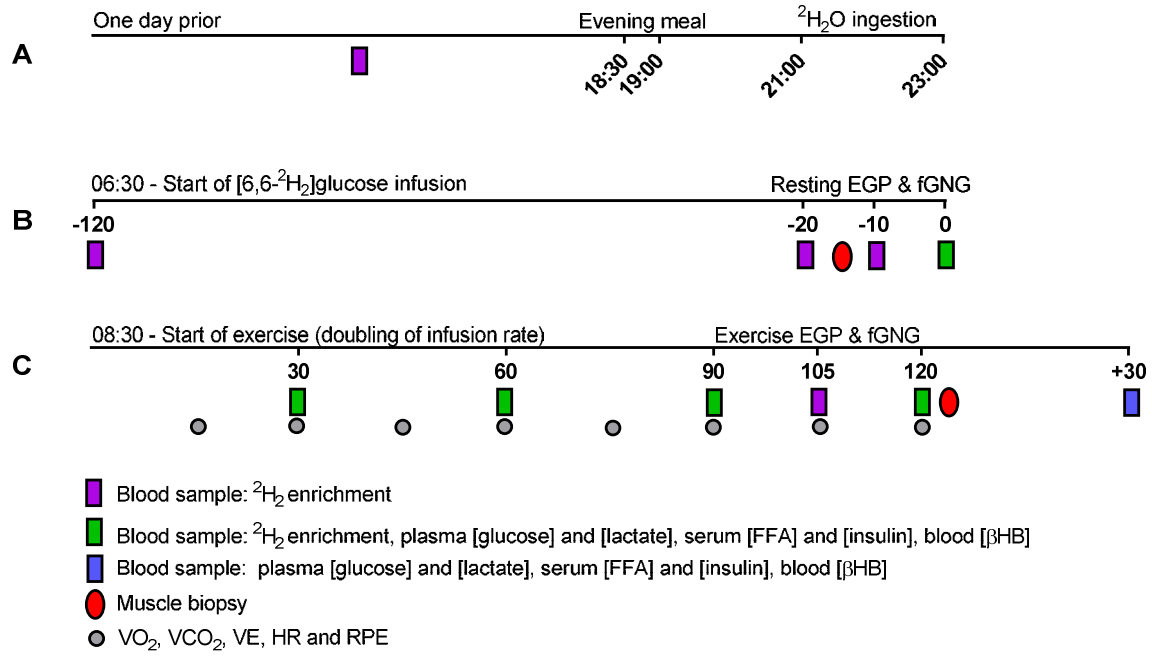

Figure 1. An overview of the protocol showing the day prior to the tracer infusion trial (A), during 2 h of rest on the morning of the trial (B) and the 2 h exercise trial at 55% of peak power output followed by 30 min of recovery (C).

2.10) Gaseous exchange, heart rate and RPE. During the last 5 min of each 15 min interval of the 2 h ride, VE, VO_2 and VCO_2 were recorded. The participants only wore the mask during this 5 min period; the first 1 min and last 15 seconds of each measurement period was excluded from the analysis to ensure that steady-state data was used. Because of the warm up, participants did not reach the full exercise intensity until half-way through the first measurement period and this time-point was therefore excluded from the analysis of respiratory data. HR data were recorded continuously and averaged during the reported minute. Rating of perceived exertion (RPE) was taken before and after the 5 min period during which respiratory measurements were recorded using the Borg 6-20 RPE scale [14].

2.11) Oral glucose tolerance. An OGTT was administered no sooner than three days after the tracer infusion trial. The OGTT was conducted in the morning after an overnight fast and participants did not participate in any physical activity the day before the OGTT. Upon arrival at the laboratory, a cannula was inserted into a forearm antecubital vein and attached to a three-way stopcock. Venous blood samples (5 ml) were obtained at -10 min and immediately before the participants ingested 75 g glucose dissolved in 250 ml of water. Blood samples were obtained at 30, 60, 90, and 120 min during the OGTT for determination of plasma glucose and serum insulin concentrations.

2.12) Blood sampling. Plasma for the determination of [6,6-$^2H_2$] glucose and ^2H_2O enrichments was collected in vacutainers spray-coated with K_2EDTA. Plasma samples for the determination of glucose and lactate concentrations were collected in vacutainers containing oxalate and fluoride. Serum samples for the determination of insulin and FFA concentrations were collected in vacutainers containing a clot activator and gel for serum separation. All tubes were inverted at least five times to ensure complete mixing with the tube additives.

All blood tubes collected during the tracer infusion trial were centrifuged immediately after collection for 10 min at 3000 rpm. Samples were then aliquoted into separate tubes for each analysis and were stored on dry-ice until the end of the exercise trial. They were then stored at either -20°C (lactate and glucose) or at -80°C (other metabolites) for subsequent analyses. The blood samples collected during the OGTT were stored on ice until the end of the test and were then processed and stored appropriately for glucose and insulin analysis. βHB concentration was determined from a drop of whole-blood using a FreeStyle Optium Xceed β-ketone meter and FreeStyle Optium H test strips. This was done immediately after the blood sample was obtained.

2.13) Muscle biopsies. A biopsy was obtained from the vastus lateralis muscle of the right leg prior to the start of exercise (~ -15 min) using the method of Bergstrom [72] as modified by Evans et al [47]. At the end of exercise, participants were immediately transferred from the bike to a plinth for a post-exercise biopsy from the vastus lateralis of the left leg. This biopsy took place between 5 min and 10 min after the cessation of exercise. Biopsy samples were quickly dissected free of connective tissue, frozen rapidly in liquid nitrogen and stored in liquid nitrogen for subsequent analyses.

2.14) Substrate Analyses. Unless specified, all sample analysis was performed by the author of this thesis. Plasma glucose and lactate concentrations were measured using the glucose oxidase method (YSI 2300 STAT PLUS, Ohio, USA). Serum insulin concentrations were measured with Automated Chemiluminescence (Centaur CP system Siemens Healthcare Diagnostics Inc. NY, USA). Glucose and insulin analyses were performed by a technician at the analytical facility at the ESSM department of UCT.

Serum FFA concentrations were determined by spectrophotometric measurements using a commercial kit (FFA half-micro test; Roche Applied Science, Mannheim, Germany). Briefly, 12.5 µl of serum sample was added to a 250 µl solution containing ATP, coenzyme A (CoA) and acyl-CoA-synthetase and incubated at 25˚C for 10 min. This activated the FFA, forming acyl-CoA. N-ethyl-maleinimide was added to remove surplus CoA before the oxidation of acyl-CoA to enoyl-CoA and H_2O_2 by acyl-CoA-oxidase. The resulting H_2O_2 converts 2,4,6-tribromo-3-hydroxy-benzoic acid and 4-aminoantipyrine to a red dye in the presence of peroxidase. The intensity of the red dye was measured in the visible wavelength range at 546 nm (BioTek Synergy HT Multi-Mode Microplate Reader, BioTek Instruments Inc, Vermont, USA).

Muscle glycogen content was measured as glucose equivalents by the glucose oxidase method following the acid hydrolysis of glycogen to glucose [121]. Briefly, approximately 20 mg wet weight of muscle was weighed. The muscle sample was alkaline digested in 200 µl of 40% KOH. Glycogen was precipitated by adding 0.8 ml absolute ethanol and incubating overnight at 4˚C. The samples were centrifuged and the glycogen pellet was isolated and hydrolised to glucose by adding 0.2 ml of 2N HCl. This was placed in a heating block at 95˚C for 3 h with occasional mixing. The pH was adjusted to approximately 7.5 using 2M NaOH and the glucose concentration of the sample was determined using the glucose oxidase method.

2.15) Isotope analysis. Plasma samples were shipped to our collaborator, Dr Chacko at the Baylor College of Medicine in Houston, Texas, and analysed by his research group according to the method described in Chacko et al. [25]. Briefly, using gas chromatography–mass spectrometry (GCMS), the pentaacetate derivative of glucose yields fragments at a mass-to-charge ratio (m/z) of 169 in unlabelled glucose. Labelled [6,6-$^2H_2$] glucose yields fragments at m/z 171 and the M+2 molar percent enrichment (MPE) was measured with m/z 171/169 [25]. The rate of appearance (Ra) of glucose in the circulation was calculated using M+2 MPE (see section 2.16). The average deuterium method was used to determine the fractional GNG [25]. For this, the pentaacetate derivative of glucose was monitored by GC-MS with m/z 170/169 to determine the M+1 enrichment of deuterium in plasma glucose carbons (C-1, 3, 4, 5, 6, 6) [25]. Deuterium enrichment in plasma water (E^2H_2O) was determined by isotope ratio mass spectrometry (Delta+XL IRMS; Thermo Finnigan, Bremen, Germany) [25]. Fractional GNG was calculated using M+1 MPE and E^2H_2O (see section 2.16).

2.16) Calculations.

Body fat (%) was calculated using the following equations:

$$\text{BD} = 1.112 - 0.0004349 \times \text{SS} + 0.00000055 \times \text{SS}^2 - 0.00028826 \times \text{y} \quad [73]$$

$$\text{Body fat (\%)} = (495/\text{BD}) - 450 \quad [142]$$

Where BD is body density, SS is the sum of seven skinfolds (mm) and y is age (years).

CHO and fat oxidation rates and energy expenditure were calculated from $\dot{V}O_2$ and $\dot{V}CO_2$ using stoichiometric equations [50] and appropriate energy equivalents, with the assumption that urinary nitrogen excretion rate was negligible:

$$\text{CHO oxidation rate (g/min)} = 4.55 \times \text{VCO}_2 - 3.21\ \text{VO}_2$$

$$\text{Fat oxidation rate (g/min)} = 1.67 \times \text{VO}_2 - 1.67\ \text{x}\ \text{VCO}_2$$

Ra glucose was calculated during the last 20 min of rest (-20, -10 and 0 min), and Ra glucose and the rate of disappearance from the circulation (Rd) glucose were calculated during the last 30 min of exercise (90, 105 and 120 min) using the non-steady-state equations of Steele modified for use with stable isotopes [162]:

$$\mathrm{Ra} = \frac{\mathrm{F} - \mathrm{V}\left(\frac{\mathrm{C1}+\mathrm{C2}}{2}\right)\left(\frac{\mathrm{IE2}-\mathrm{IE1}}{\mathrm{t2}-\mathrm{t1}}\right)}{\frac{\mathrm{IE1}+\mathrm{IE2}+\mathrm{IE3}}{2}}$$

$$\mathrm{Rd} = \mathrm{Ra} - \mathrm{V}\left(\frac{\mathrm{C2}-\mathrm{C1}}{\mathrm{t2}-\mathrm{t1}}\right)$$

Where F is the infusion rate (mg/kg/min), V is the blood pool (0.18 l/kg), C is the plasma concentration of glucose (mg/dl), IE is the M+2 enrichment (MPE) and t is time (min). The infusion rate was then subtracted from Ra to calculate the rate of EGP:

$$\mathrm{EGP} = \mathrm{Glucose\ Ra} - \mathrm{infusion\ rate}$$

The average enrichment of deuterium on each glucose carbon was calculated with the following equation [25]:

$$\mathrm{Average\ enrichment} = (\mathrm{M}+1)/6$$

Where M+1 is the deuterium enrichment of glucose using m/z 170/169 and '6' is the number of ^{2}H labelling sites on the m/z 169 fragment of glucose [25]. Since body water is the precursor pool for deuterium and hydrogen during GNG, if 100% of glucose was derived from GNG the average deuterium enrichment of glucose carbons would equal the deuterium enrichment of body water. Therefore, assuming steady-state conditions, fractional GNG is calculated as follows [25]:

$$\text{Fractional GNG} = \text{average enrichment} / E^2H_2O$$

Where E^2H_2O is the deuterium enrichment in body water.

Insulin resistance was calculated according to the reduced homeostatic model assessment (HOMA-IR) [153] with the following equation:

$$\text{HOMA-IR} = (\text{FPG x FPI}) / 22.5$$

Where FPG is fasting plasma glucose (mmol/l) and FPI is fasting plasma insulin (U/l). The Matsuda index of insulin sensitivity [102] was calculated as follows:

$$\text{Matsuda index} = \frac{10\,000}{\sqrt{(\text{FGP} \times \text{FPI}) \times (\bar{G} \times \bar{I})}}$$

Where FPG is fasting plasma glucose (mg/dl), FPI is fasting plasma insulin (U/l), $\bar{G}$ is the mean plasma glucose concentration during the OGTT (mg/dl) and $\bar{I}$ is the mean plasma insulin concentration during the OGTT (U/l).

2.17) Statistical analyses. All data are expressed as the mean ± SD unless otherwise stated. Normality was tested using Shapiro-Wilk's W test. Where normally distributed, differences between groups were detected using the independent t-test. Where not normally distributed the Mann-Whitney U test was used. Two-way ANOVA for repeated measures was used to determine differences in substrate oxidation rates and hormone and substrate concentrations during the 2 h tracer infusion trial and OGTT as well as for differences between rest and exercise in EGP and muscle glycogen content. Where a significant interaction effect was detected, further statistical analysis was performed using the Tukey HSD post-hoc test. The Chi-square test was used to determine differences in the fractional contribution of GNG to EGP (fractional GNG). A dependent t-test was used to determine differences in Ra and Rd within each group. Pearson's r was used to calculated R^2 and p values for correlations. Differences were considered significant at p values < 0.05. Statistical analysis was performed using Statsoft's Statistica 12 software.

3) RESULTS

3.1) Participant and dietary characteristics. Our advertisements were designed to recruit middle-aged, competitive male cyclists who followed a self-selected LCHF or high-CHO diet for longer than six months. Potential participants were screened for diet eligibility using a three-day dietary record which was then repeated during the three days prior to the tracer infusion trial (reported data). Despite initially attempting to recruit a high-CHO control group, in line with the recommended CHO intake for competitive endurance athletes, only one participant screened for the high-CHO group (a vegetarian) met these criteria and habitually ate more than 60% of his calories as CHO. Based on this finding, we included participants that ate approximately 50% of their calories as CHO and assigned the term 'mixed diet' as the control group. As intended, participants in each diet group were well matched for age, weight, BMI, body fat percentage, PPO and VO_{2max} (Table 1). The range of ages was 29 to 44 years in the LCHF group and 24 to 40 years in the mixed diet group. The macronutrient composition of the diets during the three days prior to the tracer infusion trial consisted of 21% protein, 72% fat and 7% CHO on the LCHF diet; 16% protein, 33% fat and 51% CHO on the mixed diet ($p < 0.01$ for differences in the proportion of fat and CHO between groups). The habitual LCHF diet contained approximately 50 g of total CHO (including dietary fibre) per day (Table 2).

Table 1. Participant characteristics for the LCHF and mixed diet groups

Variable	LCHF	Mixed	p value
Age (y)	36 ± 6 (29 – 44)	32 ± 5 (24 – 40)	0.24
Weight (kg)	78 ± 9 (65 – 90)	74 ± 8 (62 – 89)	0.41
BMI (kg/m^2)	23.6 ± 1.8 (20 – 27)	23.4 ± 2.0 (20 -27)	0.76
Body fat (%)	10 ± 3 (7 – 14)	10 ± 3 (7 – 17)	0.90
HR_{max} (beats/min)	184 ± 5 (176 – 193)	182 ± 8 (172 – 193)	0.54
VO_{2max} (ml/min)	4683 ± 445 (4019 – 5388)	4573 ± 483 (3770 – 5054)	0.69
VO_{2max} (ml/kg/min)	61 ± 5 (52 – 68)	63 ± 8 (46 – 73)	0.61
PPO (W)	369 ± 27 (322 – 422)	367 ± 38 (291 – 412)	0.93
PPO (W/kg)	4.8 ± 0.4 (4.0 – 5.2)	5.0 ± 0.4 (4.3 – 5.4)	0.34

Values are mean ± SD (range); n = 7 per group. BMI, body mass index; HR_{max}, maximum heart rate; VO_{2max}, maximal oxygen consumption; PPO, peak power output. Data were normally distributed and the p values were determined using independent t-tests.

Table 2. Dietary characteristics for the LCHF and mixed diet groups

Variable	LCHF	Mixed	p value
Time on diet (months)	13 ± 6 (8 – 24)	107 ± 115 (9 – 360)	0.18
Energy intake (kcal/d)	2866 ± 296 (2310 – 3190)	3187 ± 941 (2387 – 4916)	0.41
Protein (g/d)	147 ± 35 (78 – 192)	131 ± 51 (77 – 194)	0.50
Fat (g/d)	231 ± 21 (201 – 256)	120 ± 52 (65 – 216)	**< 0.01**
CHO (g/d)	50 ± 20 (15 – 82)	394 ± 102 (272 – 561)	**< 0.01**
Protein (g/kg/d)	1.9 ± 0.5 (1.0 – 2.4)	1.8 ± 0.8 (1.0 – 2.6)	1.00
Fat (g/kg/d)	3.0 ± 0.5 (2.5 – 4.0)	1.6 ± 0.8 (0.9 – 3.1)	**< 0.01**
CHO (g/kg/d)	0.7 ± 0.3 (0.2 – 1.2)	5.5 ± 1.9 (3.1 – 8.1)	**< 0.01**

Values are mean ± SD (range); n = 7 per group; Where data were normally distributed the p values were determined using the independent t-test, where not normally distributed the p values were determined using the Mann-Whitney U test.

The diets of both groups were similar in terms of daily caloric intake (Table 2), even when normalised to body weight (p = 0.31) (Figure 2). Daily protein intake was similar between groups, making fat and CHO consumption the only significant differences in macronutrient intake (p < 0.01) (Figure 2). Sporting nutritional guidelines often express macronutrient intake relative to body weight. Expressed this way, protein intake was similar between groups (1.9 g/kg/d on the LCHF diet and 1.8 g/kg/d on the mixed diet) and CHO intake was 0.7 ± 0.3 g/kg/d in the LCHF group compared to 5.5 ± 1.9 g/kg/d (p < 0.01) in the mixed diet group (Table 2). The participant with the highest fat intake in the mixed diet group ate approximately 3 g/kg/d of fat and had the highest total calorie intake of all the participants.

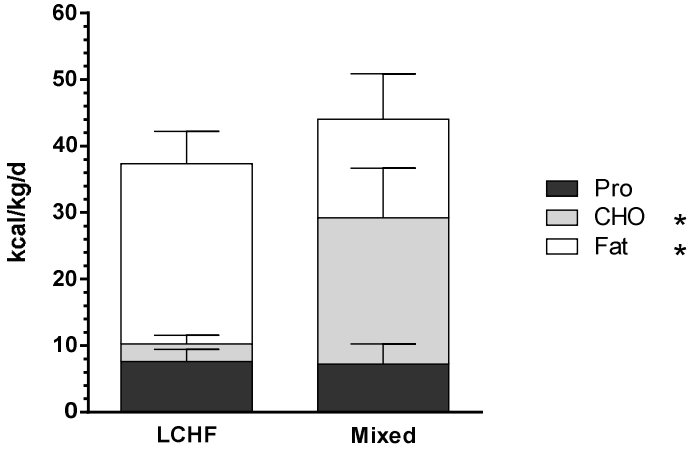

Figure 2. Macronutrient intake expressed as calories per kilogram body weight per day. * denotes a significant difference between groups (p < 0.01). *Where data were normally distributed the p values were determined using the independent t-test, where not normally distributed the p values were determined using the Mann-Whitney U test*

A detailed breakdown of nutrient intake shows that despite the LCHF group eating almost twice the amount of fat, the intake of polyunsaturated fatty acid (PUFA) did not differ in the LCHF and mixed diet groups (Table 3). The extra fat consumed by the LCHF group was primarily saturated and monounsaturated fatty acids (MUFA), while sugar and fibre consumption was higher in the mixed diet (Table 3). To avoid over-interpretation, vitamin and mineral intakes were not reported here as the validity of the micronutrient data was uncertain. Foods which were present in the diets of the majority of participants and/or contributed towards a large proportion of caloric intake are listed in Table 4, along with the main sources of CHO in the LCHF group and the main sources of fat in the mixed diet group. The mixed diet group commonly consumed processed foods, such as bread, muesli, ProNutro/FUTURELIFE (breakfast cereal/porridge), pasta, low-fat yoghurts and fruit juice.

Table 3. Sugar, fibre and fatty acid profile of the LCHF and mixed diet groups

Variable	LCHF	Mixed	p value
Sugar (g/d)	25 ± 15	173 ± 60	**< 0.01**
Fibre (g/d)	13 ± 6	42 ± 17	**< 0.01**
Saturated fat (g/d)	97 ± 11	39 ± 15	**< 0.01**
MUFA (g/d)	90 ± 20	44 ± 20	**< 0.01**
PUFA (g/d)	26 ± 7	26 ± 15	0.91

Values are mean ± SD; n = 7 per group; Where data were normally distributed the p value was determined using the independent t-test, where not normally distributed the p value was determined using the Mann-Whitney U test.

Table 4. Foods commonly consumed by the LCHF and mixed diet groups.

LCHF		**Mixed**	
Common Foods	Main sources of CHO	Common Foods	Main sources of fats
Butter	Avocado	ProNutro/FUTURELIFE	Mayonnaise
Coconut oil	Mixed salad greens	Muesli/Oats	Eggs
Cream	Tomatoes	Whole-wheat bread	Cheese
Beef (steaks/biltong)	Squash	Milk	Muesli/Oats
Pork (crackling/bacon)	Spinach	Chicken breast	Seeds
Eggs	Carrots	Eggs	Beef
Fish	Mushrooms	Pasta	Lamb
Cheese	Cabbage	Yoghurt	Olive oil
Nuts	Green beans	Fruit juice	Milk
Olive oil	Cocoa powder	Fruit	Nuts

3.2) Exercise intensity. Heart rate (HR), oxygen consumption (VO_2) and rating of perceived exertion (RPE) was recorded during the last 5 min of every 15 min period during the 2 h ride. The intensity of the ride was standardised to 55% of PPO which was 203 ± 16 W and 202 ± 23 W for the LCHF and mixed diet groups respectively (p = 0.93). Exercise intensity was similar between groups in terms of $\%HR_{max}$ and $\%VO_{2max}$ (Figure 3A & 3B) and during the final 30 min of the ride, when EGP and fractional GNG was measured, the intensity of the ride was 87 ± 4 $\%HR_{max}$ and 72 ± 5 $\%VO_{2max}$ in the LCHF group and 90 ± 4 $\%HR_{max}$ and 72 ± 7 $\%VO_{2max}$ in the mixed diet group. RPE was similar between groups and there was no difference in perception of effort at any stage during the ride (Figure 3C).

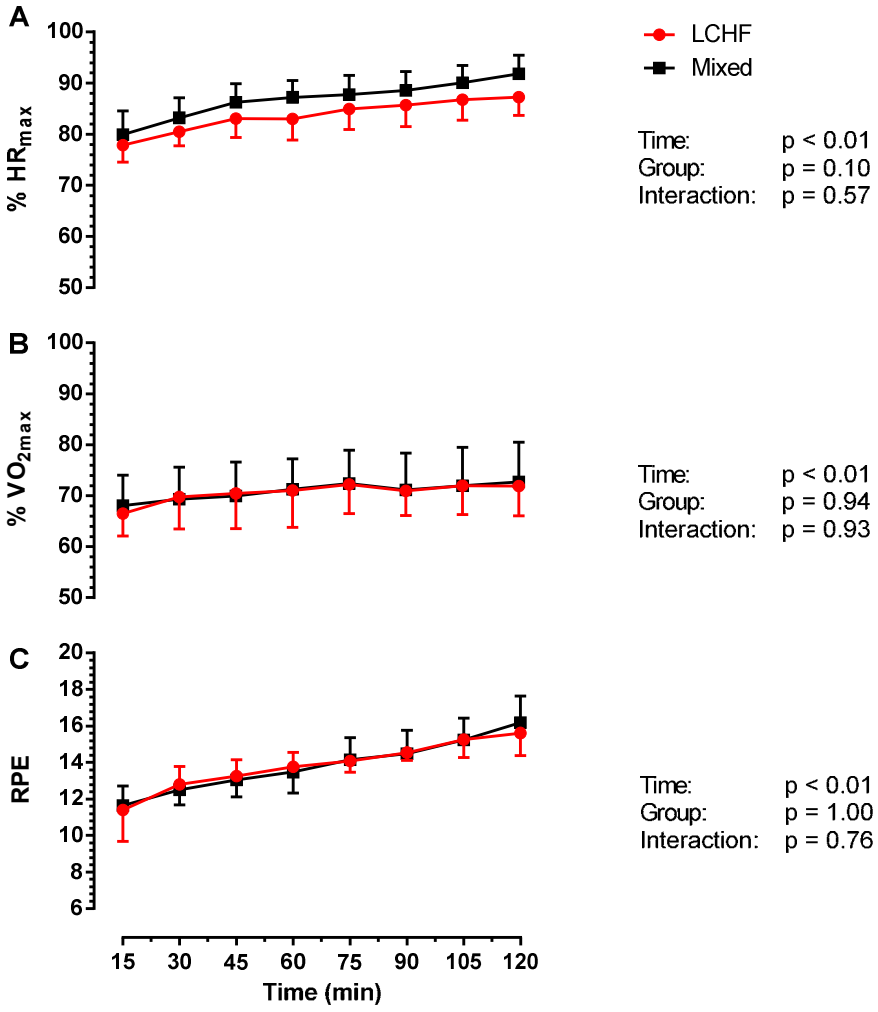

Figure 3. Percentage of maximum heart rate (A), percentage of maximum oxygen consumption (B) and rating of perceived exertion (C) during the 2 h submaximal ride at 55% of peak power output. Data presented as mean ± SD; n = 7 per group; p values were determined using repeated measures ANOVA.

3.3) Indirect calorimetry. Substrate utilisation and energy expenditure was estimated from VO_2, VCO_2 and expired ventilation (VE) which was measured during the last 5 min of every 15 min period during the 2 h ride. Energy expenditure during the 2 h ride was not different between groups and was 1853 ± 174 kcal in the LCHF group and 1963 ± 197 kcal in the mixed diet group ($p = 0.29$). Respiratory exchange ratio (RER) and CHO oxidation rates were significantly lower in the LCHF group than in the mixed diet group but fat oxidation rates were significantly higher in the LCHF group (Figure 5A, 5B & 5C). In the mixed diet group there was a gradual decrease in RER and increase in fat oxidation rate as exercise progressed. This did not occur in the LCHF group in which the RER was unchanged from 30 min to the end of exercise. The average CHO oxidation rate over the 2 h ride was 5.1 ± 1.9 kcal/min (1.3± 0.5 g/min) and 12.0 ± 2.2 kcal/min (3.0 ± 0.5 g/min) on the LCHF and mixed diets respectively; while average fat oxidation rates were 10.5 ± 1.8 kcal/min (1.2 ± 0.2 g/min) and 4.3 ± 2.0 kcal/min (0.5 ± 0.2 g/min) on the LCHF and mixed diet respectively. The individual with the highest rate of fat oxidation in the mixed diet group and the individual with the lowest rate of fat oxidation in the LCHF group had RER, CHO oxidation rates and fat oxidation rates which partially overlapped (Figure 5D, 5E & 5F). Apart from these two participants, there was no overlap in RER or in rates of CHO or fat oxidation between individuals within either group. The individual with the highest rate of fat oxidation in the LCHF group averaged 1.45 g/min from 30 min to 120 min.

The LCHF participants used 140 ± 23 g of fat (1.8 ± 0.2 g/kg) and 148 ± 57 g of CHO (1.9 ± 0.7 g/kg) to fuel their rides, with fat accounting for 68 ± 11 % of total energy expenditure. The mixed diet group used 58 ± 25 g of fat (0.8 ± 0.3 g/kg) and 361 ± 62 g of CHO (4.9 ± 0.9 g/kg), with fat accounting for 26 ± 10 % of total energy expenditure ($p < 0.01$)(Figure 4).

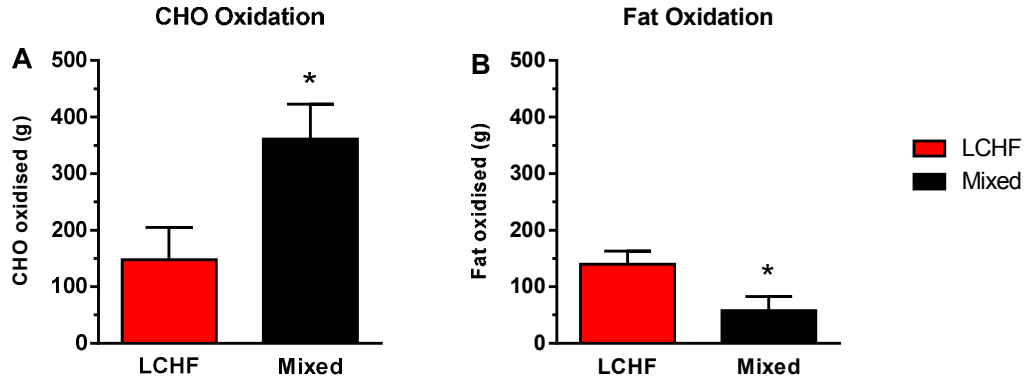

Figure 4. Total carbohydrate (A) and fat (B) oxidised during the 2 h ride. Data presented as mean ± SD; n = 7 per group; * denotes a significant difference between groups ($p < 0.01$). p values were determined using the independent t-test.

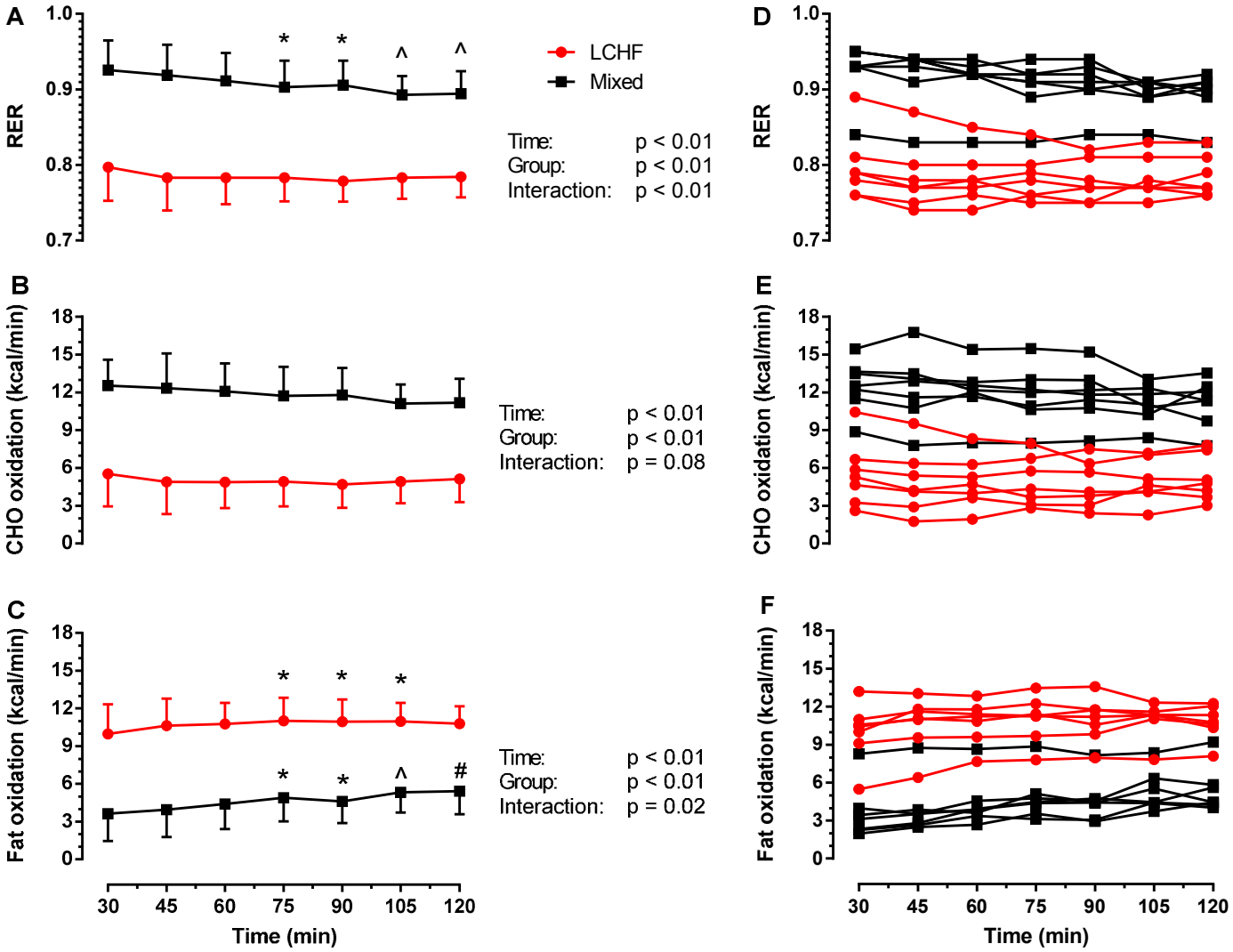

Figure 5. Respiratory exchange ratio (A), rate of CHO oxidation (B) and rate of fat oxidation (C) during steady-state exercise at 55% of peak power output. Data presented as mean ± SD; n = 7 per group; * denotes a significant difference to 30 min; ^ denotes a significant difference to 30 and 45 min; # denotes a significant difference to 30, 45 and 60 min. p values were determined using repeated measures ANOVA and significant differences marked on the figure were determined using Tukey HSD post-hoc test. Values for each individual's respiratory exchange ratio (D), CHO oxidation rate (E) and fat oxidation rate (F).

3.4) Plasma glucose concentrations. Blood samples were drawn every 30 min during exercise and after 30 min of recovery. During exercise there was no difference in plasma glucose concentration between groups (Figure 6A). Blood glucose concentrations were significantly lower after 90 and 120 min of exercise compared to baseline but were still within the normal range (4.6 ± 1.0 mmol/l). Plasma glucose concentration in one participant in the LCHF group fell to 3 mmol·L^{-1} after 60 min of exercise, which would be considered close to hypoglycaemia (Figure 6B). However, his RPE was 13 and did not increase at this point compared to the average of all participants. He did not experience any symptoms of hypoglycaemia and his plasma glucose concentration increased throughout the remainder of the trial. There was one case in each group in whom plasma glucose concentrations increased at the start of exercise and remained above 6 mmol/l throughout the 2 h ride. Every individual in the LCHF group had an increase in plasma glucose concentrations during 30 minutes of recovery (Figure 6B) which was not seen in the mixed diet group (Figure 6C).

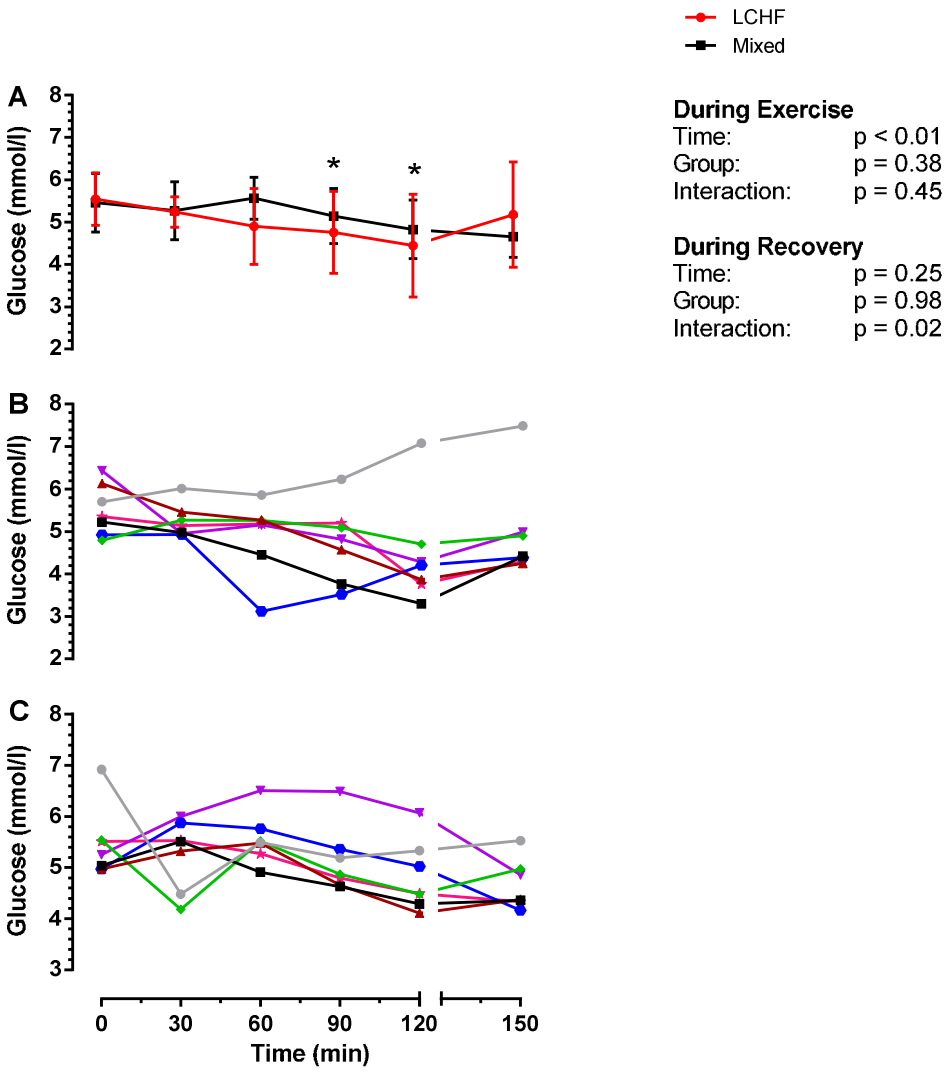

Figure 6. Group plasma glucose concentrations during the 2 h submaximal ride at 55% of peak power output and after 30 minutes of recovery (A). Values are presented as means ± SD; n=7 per group; * denotes a significant difference in plasma glucose concentration compared to 0 min. p values were determined using repeated measures ANOVA. Values for each individual's plasma glucose concentration in the LCHF group (B) and mixed diet group (C). To convert mmol/l to mg/dl multiply by 18.

3.5) Deuterium enrichment. Participants drank 4 g/kg body weight (99% atom % ^2H) ^2H_2O the night before the tracer trial with the intention of enriching body water to 0.5% ^2H_2O. Suitable body water ^2H_2O enrichment was achieved in all participants (Table 5) and the mean enrichment in each individual did not fluctuate throughout the 4 h duration of the trial. Fractional GNG was calculated from the M+1 MPE of glucose carbons (Figure 7A & 7B) and the rate of EGP was calculated from the M+2 MPE of [6,6-$^2H_2$]glucose (Figure 7C & 7D). Resting measurements were based on the isotope enrichments obtained at -20, -10 and 0 min and exercise measurements were based on isotope enrichments obtained at 90, 105 and 120 min.

Table 5. Average deuterium enrichment of body water in each individual during the 4 h tracer infusion trial (-120 min to 120 min)

LCHF (%)	0.50 ± 0.00	0.51 ± 0.00	0.48 ± 0.00	0.53 ± 0.00	0.55 ± 0.00	0.56 ± 0.00	0.50 ± 0.00
Mixed (%)	0.45 ± 0.01	0.56 ± 0.01	0.49 ± 0.00	0.56 ± 0.00	0.52 ± 0.01	0.53 ± 0.00	0.53 ± 0.01

Values are mean ± SD

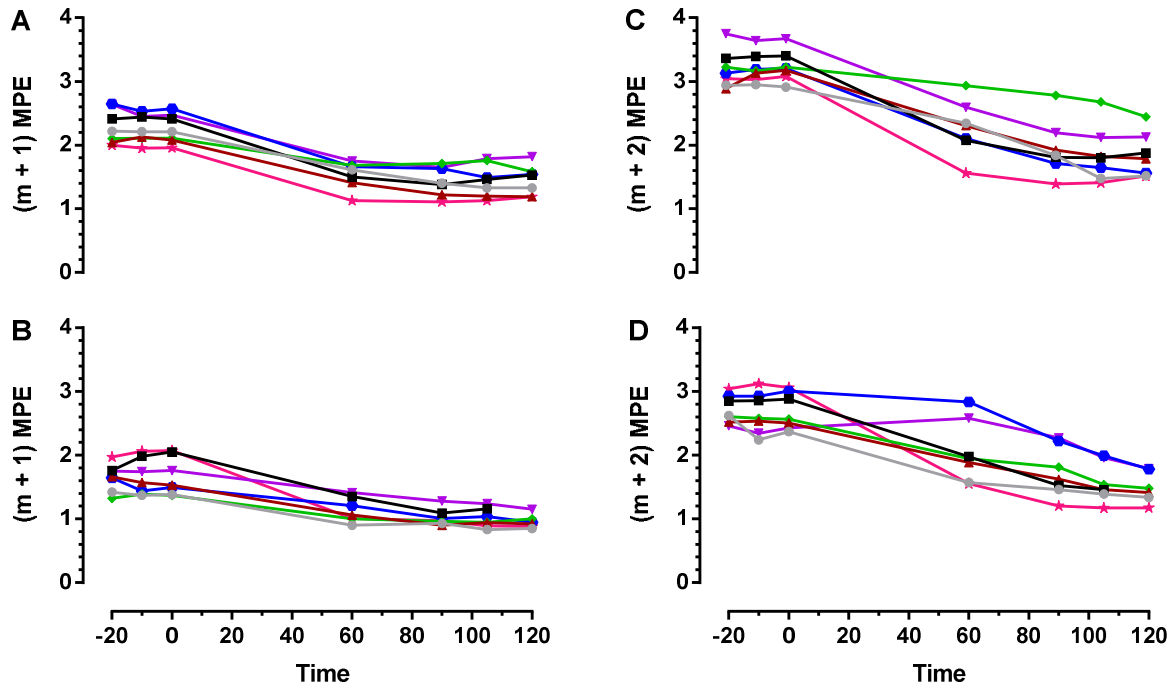

Figure 7. M+1 mole percent excess (MPE) of glucose for individuals in the LCHF group (A) and mixed diet group (B) and M+2 MPE of glucose for individuals in the LCHF group (C) and mixed diet group (D).

3.6) Resting EGP and fractional GNG. The absolute rate of GNG prior to exercise was similar between groups ($p = 0.19$) while the rate of GLY was significantly higher ($p < 0.01$) in the mixed diet group than in the LCHF group (Figure 9A). Total EGP was higher in the mixed diet group compared to the LCHF group, primarily due to the higher rate of GLY (Figure 9A). The fractional GNG was significantly ($p < 0.01$) higher in the LCHF group compared to the mixed diet group at rest (Figure 8A), which was due to differences in GLY between groups and not to an increase in the rate of GNG in the LCHF group.

3.7) Change in EGP and fractional GNG from rest to exercise. The fractional GNG fell significantly and similarly in both groups from rest to exercise by around 0.23, indicating that the proportional contribution of GNG to EGP decreased by 23% from rest to exercise (Figure 10A). This occurred despite a 2.4-fold average increase in the absolute rate of GNG from rest to exercise in both groups (Figure 10B). The absolute increase in GLY was significantly larger in the mixed diet group than the LCHF group, with GLY increasing by 4.3 ± 1.0 mg/kg/min compared to 2.8 ± 0.6 mg/kg/min (Figure 10C). This represented a 7.3-fold increase in GLY in the LCHF and a 5.5-fold increase in the mixed diet group. The rate of EGP increased to a greater extent in the mixed diet group (5.8 mg/kg/min) than in the LCHF group (4.4 mg/kg/min), mainly due to the larger increase in GLY in the mixed diet group (Figure 10D). There was a 3.8-fold increase in EGP in the LCHF group and a 3.9-fold increase in EGP in the mixed diet group.

3.8) EGP and fractional GNG during exercise. There was no difference between groups in the absolute rate of GNG during exercise ($p=0.15$) (Figure 9B). The rate of GLY was significantly higher in the mixed diet group during exercise ($p < 0.01$) as was the rate of EGP ($p < 0.01$)(Figure 9B). The fractional GNG was significantly higher ($p = 0.04$) in the LCHF group compared to the mixed diet group during exercise, which was primarily attributable to differences in GLY (Figure 8B). There was a strong correlation between the rate of GLY and EGP but no correlation between GNG and EGP (Figure 11C and 11D). The rate of EGP in the LCHF group was of 6.0 ± 0.9 mg/kg/min compared to 7.8 ± 1.1 mg/kg/min in the mixed diet group ($p < 0.01$). Glucose Rd during exercise was 6.4 ± 1.0 mg/kg/min in the LCHF group and 8.2 ± 1.0 in the mixed diet group ($p = 0.02$). There was no difference between EGP and Rd within the LCHF group ($p = 0.38$ dependent t-test) but Rd was significantly higher than EGP within the mixed diet group ($p < 0.01$ dependent t-test). EGP and Rd were highly correlated (Figure 11B).

3.9) Glucose production relative to glucose oxidation. Glucose Rd was significantly correlated with the rate of CHO oxidation during exercise (Figure 11A). The rate at which glucose was produced via GNG was approximately 20% of the estimated rate of total CHO oxidation in the LCHF group during the final 30 min of exercise (Table 6). This is over three times greater than the percentage of CHO oxidation that GNG contributes in the mixed diet group ($p < 0.01$) (Table 6). Total EGP could provide approximately 42% of glucose oxidation requirements during exercise in the LCHF group, which is significantly higher than the 20% estimated for the mixed diet group ($p < 0.01$) (Table 6).

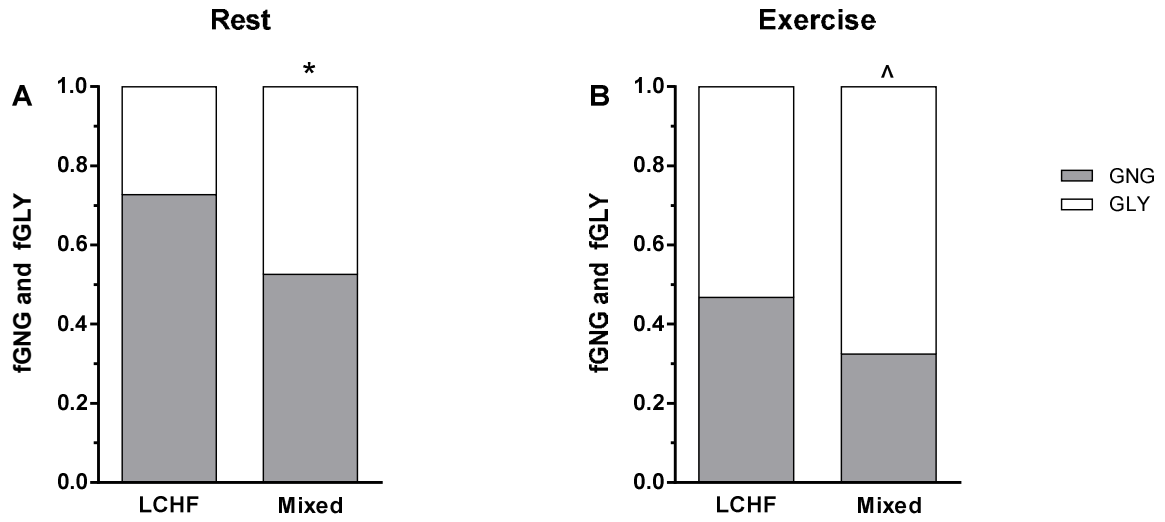

Figure 8. The fractional contribution of gluconeogenesis and glycogenolysis to endogenous glucose production during steady-state at rest (A) and during exercise (B). n = 7 per group; * denotes a significant difference between groups in the fractional contribution of GNG and GLY ($p < 0.01$). ^ denotes a significant difference in the fractional contribution of GNG and GLY ($p < 0.05$). p values were determined using Chi-square test.

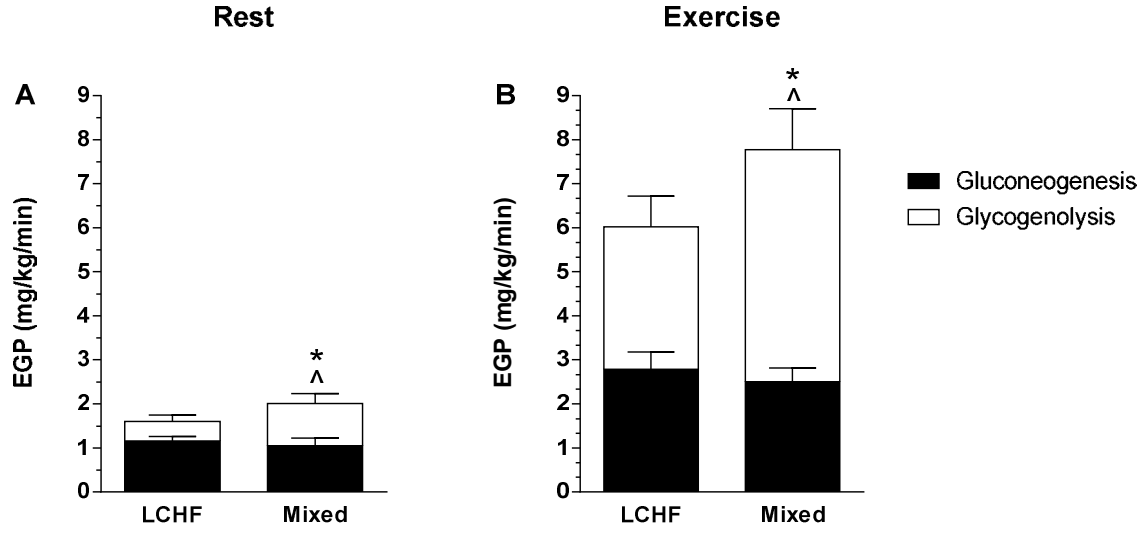

Figure 9. Total endogenous glucose production rate shown as the sum of gluconeogenesis and glycogenolysis during steady-state at rest (A) and during exercise (B). Data presented as mean ± SD; n = 7 per group; * denotes a significant difference between groups in total endogenous glucose production ($p < 0.01$). ^ denotes a significant difference in glycogenolysis ($p < 0.01$). p values were determined using an independent t-test.

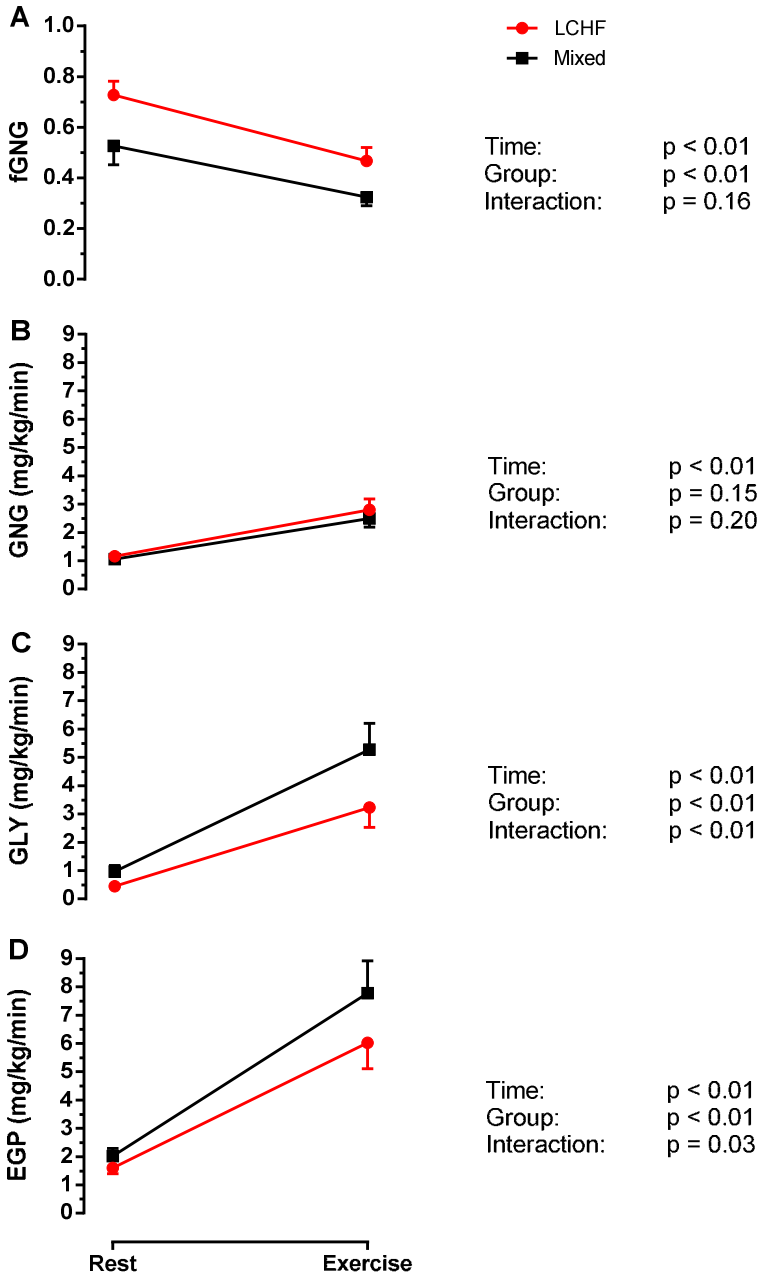

Figure 10. Comparison between rest and exercise in steady-state of the fractional contribution of gluconeogenesis to total glucose production (A), gluconeogenesis (B), glycogenolysis (C) and endogenous glucose production (D). Values are presented as means ± SD; n=7 per group. p values where determined using repeated measures ANOVA.

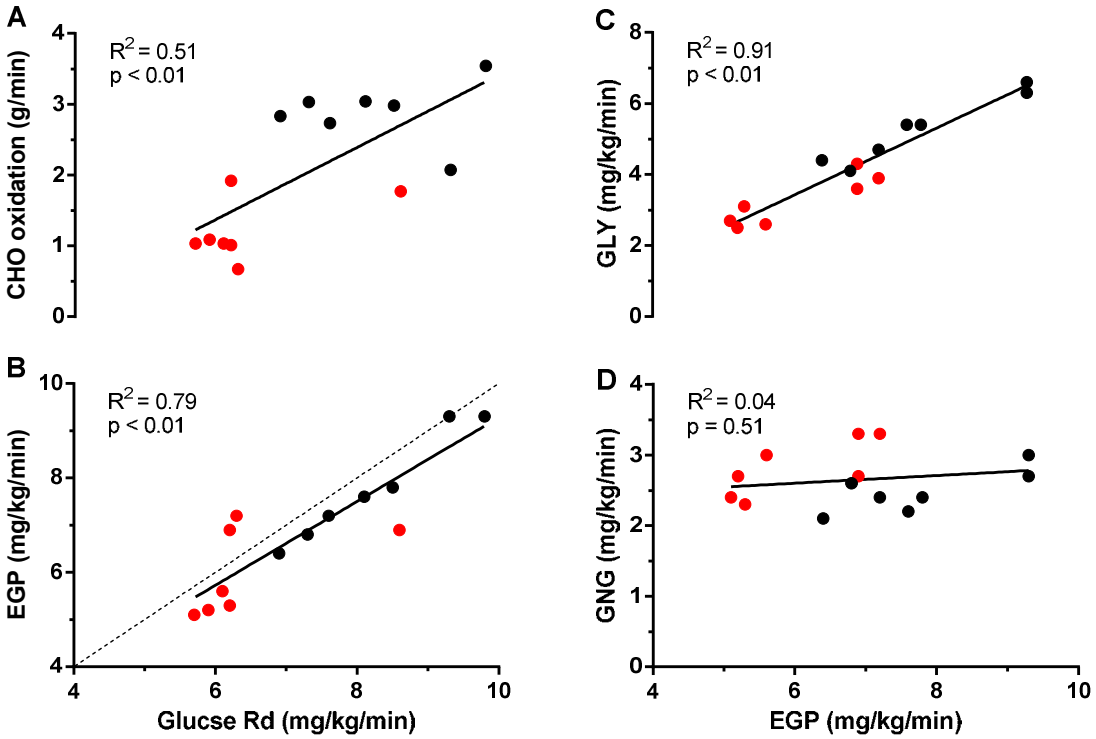

Figure 11. Correlations between CHO oxidation rate during the final 30 min of exercise and glucose rate of disappearance (A); Exercise EGP and glucose rate of disappearance (B); Exercise GLY and EGP (C); and Exercise GNG and EGP (D). The LCHF group is marked in red and the mixed diet group are in black. The dashed line in B represents where glucose Rd would be equal to EGP. Values below this line indicate that EGP is lower than Rd. n=7 per group. p values and R^2 where determined using Pearson's r.

3.10) Muscle glycogen. Muscle biopsies were obtained pre- and post-exercise to determine the pre-exercise muscle glycogen content and rate of glycogen utilisation. Pre-exercise muscle glycogen content was 1.8 times greater ($p < 0.01$) in the mixed diet group than in the LCHF group (Figure 12). Both groups had a significant reduction in muscle glycogen content during exercise ($p < 0.01$) and glycogen content was similar in both groups at the end of exercise ($p = 0.98$) (Figure 12). The rate of muscle glycogen utilisation, calculated as the linear rate of utilisation from pre- to post-exercise values, was 0.36 ± 0.14 mmol/kg/min (0.06 ± 0.03 g/kg.ww/min) in the LCHF group and 0.82 ± 0.25 mmol/kg/min (0.15 ± 0.04 g/kg.ww/min) in the mixed diet group ($p < 0.01$) (Table 6). Assuming that 100% of EGP is being oxidised, the rate of muscle glycogen utilisation that would be required to meet the remainder of CHO oxidation during the final 30 min of exercise (calculated as the rate of CHO oxidation minus the rate of EGP) would be 0.78 ± 0.42 g/min in the LCHF group; compared to 2.34 ± 0.45 g/min in the mixed diet group ($p < 0.01$), a rate three times greater in the mixed diet group than the LCHF group (Table 6).

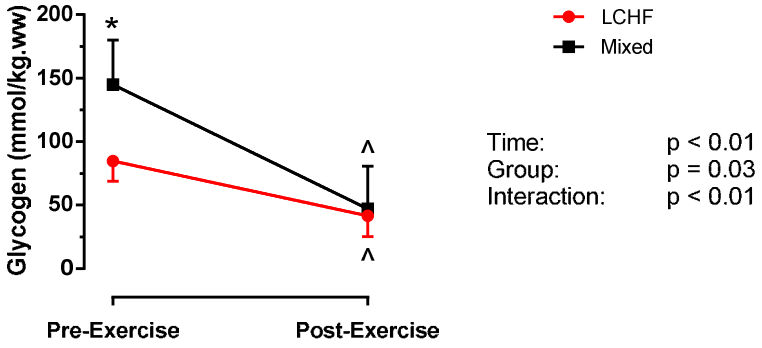

Figure 12. Muscle glycogen content pre- and post-exercise expressed per kg wet weight. Values are presented as means ± SD; n=7 per group; * denotes a significant difference to pre-exercise values in the LCHF group ($p < 0.01$). ^ denotes a significant difference to pre-exercise values of that group ($p < 0.01$). p values were determined using repeated measures ANOVA and significant differences marked on the figure were determined using Tukey HSD post-hoc test. To convert mmol/kg.ww to mg/kg.ww multiply by 180.

Table 6. Sources of glucose during exercise.

	LCHF	Mixed	p value
CHO oxidation (g/min)	1.22 ± 0.45	2.89 ± 0.44	**< 0.01**
GNG (g/min)	0.22 ± 0.04	0.18 ± 0.03	0.13
Liver GLY (g/min)	0.25 ± 0.04	0.39 ± 0.10	**< 0.01**
GNG/CHO oxidation (%)	20 ± 8	7 ± 2	**< 0.01**
EGP/CHO oxidation (%)	42 ± 16	20 ± 6	**< 0.01**
Muscle glycogen utilisation (g/kg.ww/min)	0.06 ± 0.03	0.15 ± 0.04	**< 0.01**
Muscle glycogen utilisation (g/min)*	0.97 ± 0.40	2.20 ± 0.66	**< 0.01**
CHO oxidation – EGP (g/min)	0.75 ± 0.42	2.31 ± 0.45	**< 0.01**
CHO – GNG (g/min)	1.00 ± 0.44	2.70 ± 0.45	**< 0.01**

*Values are mean ± SD; n = 7 per group; * assuming 15 kg of active muscle. Where data were normally distributed the p value was determined using an independent t-test, where not normally distributed the p value was determined using a Mann-Whitney test. CHO oxidation is the rate of CHO oxidation during the final 30 min of exercise.*

3.11) Plasma substrates and insulin concentrations. Serum FFA increased during exercise and continued to increase during recovery. However, there was no difference in serum FFA between the LCHF and mixed diet group (Figure 13A). βHB concentrations were significantly higher in the LCHF group at all time points compared to the mixed diet group, where values were close to 0 mmol/l throughout exercise (Figure 13B). In the LCHF group, blood βHB levels decreased at the onset of exercise but then gradually increased throughout exercise. There was a large increase in βHB concentration during recovery which occurred to a lesser extent in the mixed diet group. Plasma lactate concentrations increased similarly during exercise in both groups and fell during the 30 min recovery period (Figure 13C). Lactate concentrations remained relatively stable once steady-state was reached (30 min) which indicates that exercise intensity was below the lactate threshold. The plasma lactate concentration in the LCHF group was 3.2 ± 1.0 mmol/l which was similar to the 2.8 ± 1.2 mmol/l in the mixed diet group. Insulin concentrations decreased throughout exercise in both groups ($p < 0.01$) (Figure 13D) and were lower in the LCHF group ($p < 0.05$). Although statistically significant, this difference between groups was never more than 1.5 U/l throughout the exercise period, a difference that is likely without biological significance. One participant who had a vasovagal response during the pre-exercise muscle biopsy had an extremely high plasma insulin concentration at 0 min and was excluded from the analysis.

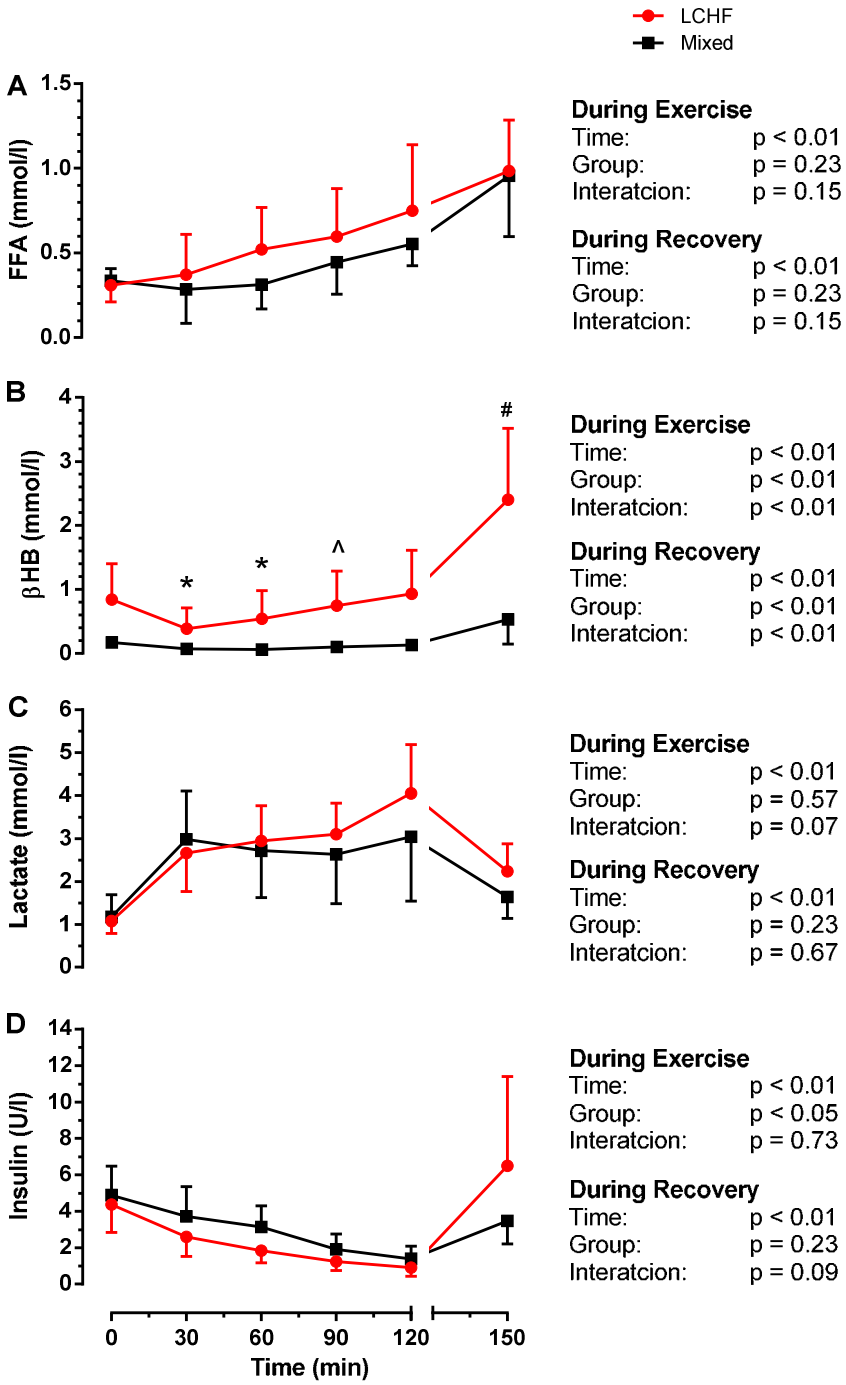

Figure 13. Plasma concentrations of free fatty acids (A), beta-hydroxbutyrate (B), lactate (C) and insulin (D). Values are presented as means ± SD; n=7 per group except for insulin where n = 6 in the mixed group. p values where determined using repeated measures ANOVA. * indicates significantly lower than 0 min and 120 min. ^ indicates significantly higher than 30 min. # indicates a significant increase from 120 min during recovery.

3.12) Oral glucose tolerance. As insulin is a key regulator of glucose homeostasis, an OGTT was administered to investigate whether there were differences in insulin sensitivities between groups. Following the ingestion of the 75 g glucose drink, plasma glucose concentrations increased to a greater extent and remained higher for longer in the LCHF group compared to the mixed diet group ($p < 0.01$) (Figure 14A). Peak insulin levels occurred after 30 min in the mixed diet group compared to 60 min in the LCHF group (Figure 13B). Insulin levels returned to values similar to baseline after 120 min in the LCHF group, compared to 60 min in the mixed diet group (Figure 14B). One participant in the LCHF group had a plasma glucose concentration of 8.14 mmol/l after 120 min which is indicative of impaired glucose tolerance. Every other individual had normal glucose tolerance according to the OGTT (120 min plasma glucose concentration < 7.8 mmol/l).

Even though there were significant differences in the glucose and insulin responses during the OGTT, there was no difference in insulin resistance between groups as classified by HOMA-IR ($p = 0.80$ Mann Whitney U) (Figure 15A). HOMA-IR considers only fasting glucose and insulin concentrations, while the Matsuda index of insulin sensitivity takes into account the glucose and insulin concentrations during the OGTT. The mixed diet group were significantly more insulin sensitive according to the Matsuda index than the LCHF group ($p = 0.01$) (Figure 15B). However, every individual is considered to have normal insulin sensitivity according to these indices and is below the HOMA-IR cut-point for insulin resistance or above the Matsuda cut-point for insulin sensitivity (Figure 14) [129].

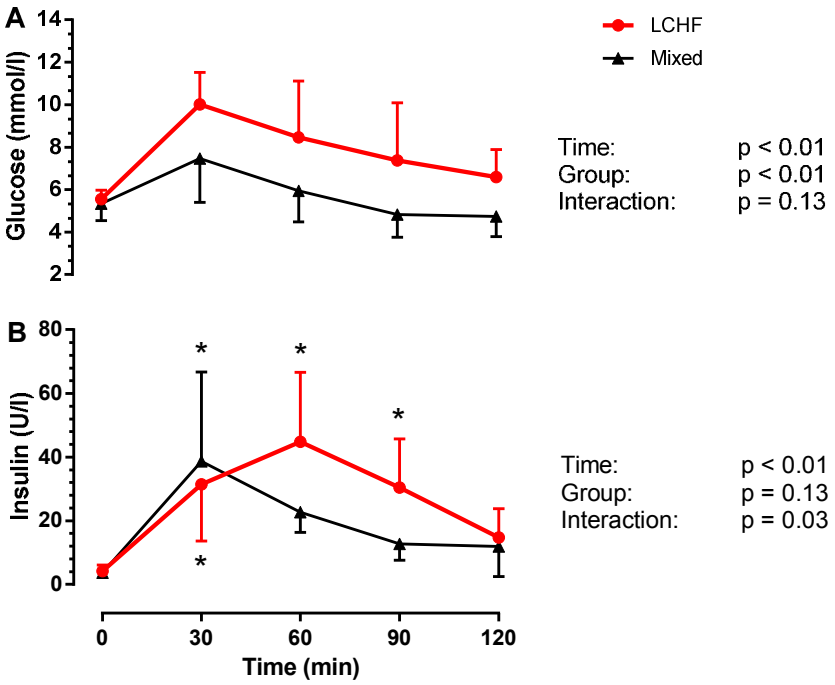

Figure 14. Concentrations of plasma glucose (A) and serum insulin (B) during a 75 g oral glucose tolerance test. Values are presented as means ± SD; n=7 per group. p values where determined using repeated measures ANOVA. * indicates a significant difference to 0 min.

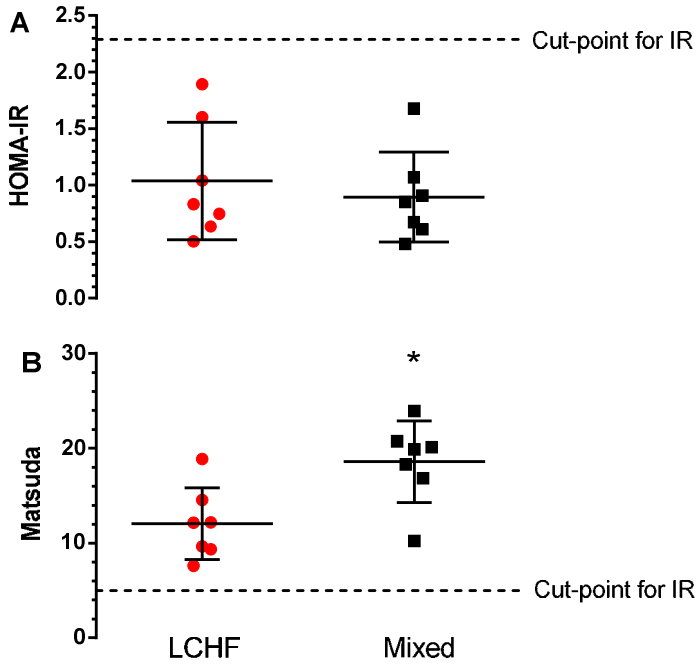

Figure 15. Indices of insulin resistance calculated from OGTT data. HOMA-IR (A) and Matsuda (B). Values are presented as means ± SD; n=7 per group. IR, insulin resistance. * denotes a significant difference between groups (p = 0.01, determined using the independent t-test). Individuals above the cut-point are considered insulin resistant for HOMA-IR and individuals below the cut-point are considered insulin resistant for Matsuda.

4) DISCUSSION

4.1) GNG and EGP. The main finding of this study was that the rate of GNG during exercise was similar in trained cyclists who habitually ate either a mixed diet or a LCHF diet (Figure 9B). The rate of EGP was significantly higher in the mixed diet group and the difference in EGP was attributable entirely to a greater rate of GLY in the mixed diet group than in the LCHF group (Figure 9B). As far as we are aware, this is the first time that the rate of GNG from all precursors has been quantified during endurance exercise in trained athletes. It is also the first time that EGP has been quantified during exercise in LCHF athletes.

Combining groups, the rate of GNG in our study was 2.6 ± 0.4 mg/kg/min during exercise. This is a higher rate of GNG than has previously been reported when GNG from only lactate or glycerol was measured [46, 152]. Emhoff et al. [46] measured the rate of GNG from lactate at 1.7 mg/kg/min during exercise at 67% VO_{2max} and 1.9 mg/kg/min at 75% VO_{2max}. Therefore, even though lactate is the primary precursor for GNG [6, 34], measuring glucose production from only lactate may underestimate the rate of total GNG by approximately 30% at exercise intensities above 65% VO_{2max}. The underestimation of GNG may be even larger over exercise periods longer than 2 h or at low intensities, in which fat oxidation rates are higher and glycerol contributes towards GNG to a greater extent [6]. At 65% VO_{2max} the rate of glycerol-derived glucose production was measured at 0.7 mg/kg/min [152], an underestimation of total GNG by approximately 70% according to our results. Therefore, studies that have quantified GNG from one precursor during exercise have underestimated the rate of total GNG and have thus overestimated the rate of GLY [46, 152].

We measured a rate of EGP of 7.8 ± 1.1 mg/kg/min at approximately 72% VO_{2max} in the mixed diet group. This is similar to the 7.6 ± 2.0 mg/kg/min at 75% VO_{2max} and 7.3 ± 2.1 at 67% VO_{2max} measured by Emhoff et al [46] in participants who ate a mixed diet. In trained athletes on a mixed diet, EGP has also been measured at 4.1 mg/kg/min at 45% VO_{2max} and 5.9 mg/kg/min at 65% VO_{2max} [152]. The rate of EGP at 72% VO_{2max} in the LCHF group was 6.0 ± 0.9 mg/kg/min which was significantly lower than that of the mixed diet group in our study (Figure 9B), but still in the same region as EGP values previously reported in athletes on a high-CHO diet exercising at 65% VO_{2max} [152]. The rate of EGP was sufficient in both the LCHF and mixed diet group to maintain plasma glucose concentration within a normal range during the 2 h ride, but not sufficient to prevent a significant reduction in plasma glucose concentration over time (Figure 6A).

After an overnight fast, LCHF athletes are therefore able to sustain a relatively high rate of EGP without relying on GNG to any greater extent than do athletes who habitually eat more CHO. Even though the rate of GLY was 1.6 times greater in the mixed diet group than in the LCHF group, a surprisingly large proportion of EGP in the LCHF group was derived from GLY (just over 50%). Since LCHF adaptation up-regulates non-oxidative glucose disposal pathways and decreases oxidative glucose disposal pathways [11, 41], a likely source of this glycogen content is from GNG at rest. Thus we show that after long-term adaptation to a LCHF diet, liver glycogen content is still sufficient to make a meaningful contribution towards EGP during exercise at 72% VO_{2max}.

In addition, we quantified EGP, GNG and GLY at rest. We found that the rate of GNG at rest was similar in athletes on either long-term LCHF or mixed diets (Figure 9A). As occurred during exercise, the rate of EGP was significantly higher in the mixed diet group, which was entirely due to a higher rate of GLY (Figure 9A). This is in agreement with the study of Bisschop et al. [12] who found that GNG was relatively stable across a wide range of CHO and fat intakes, but GLY was variable and positively associated with CHO intake. In our study the rate of EGP at rest was 1.6 ± 0.2 mg/kg/min in the LCHF group and 2.0 ± 0.3 in the mixed diet group. In the study of Bisschop et al. [12], EGP was 1.7 ± 0.1 after 11 days on a LCHF diet and 2.1 ± 0.1 mg/kg/min after 11 days on a mixed diet [12]. Acute adaptation to a LCHF diet therefore produced a similar effect on EGP as does long-term adaptation. Or stated differently, EGP is rapidly affected by a LCHF diet. Since the lower EGP after a LCHF diet is due to a lower rate of GLY, the change probably occurs as soon as liver glycogen content is reduced.

Bisschop et al. [12] measured GNG from all precursors using a similar technique to that employed in the current study [94]. The rate of GNG after long-term adaptation to a LCHF diet from the current study was 1.2 ± 0.1 mg/kg/min, very similar to values of 1.1 ± 0.0 mg/kg/min reported by Bisschop et al. [12] after 11 days of adaptation to a LCHF diet. However, we did not find the small but significantly higher rate of GNG in the LCHF group, as was reported by Bisschop et al [12]. The small difference in GNG may have been part of an acute response to the LCHF diet, which might have stabilised after the long-term adaptation studied in our experiment. Our results support the opinions of Bisschop et al. [12] and Nuttall et al. [116], that the rate of GNG at rest is relatively stable across a wide range of dietary conditions and that changes in EGP are mainly due to differences in GLY. We can extend this hypothesis to include exercise, during which GNG was relatively stable despite different metabolic and dietary statuses, whereas GLY modulated changes in the rate of EGP as a result of different habitual CHO intakes.

4.2) CHO oxidation and EGP/GNG regulation. We found significantly higher rates of CHO oxidation in the mixed diet group than in the LCHF group. The average CHO oxidation rate during the 2 h ride, as estimated using indirect calorimetry, was 1.3 ± 0.5 g/min in the LCHF group and 3.0 ± 0.5 g/min in the mixed diet group (Figure 5B). Using similar methods, Lambert et al. [93] and Zajac et al. [163] measured CHO oxidation rates of approximately 1.4 g/min and 1.3 g/min in fasted LCHF athletes during exercise. However, Phinney et al. [126] reported a CHO oxidation rate of 0.25 g/min during exercise at 64% VO_{2max} after adaptation to a ketogenic diet. It has been proposed that the very low CHO oxidation rates reported by Phinney et al. [126] may have been due to the production of ketones without their subsequent oxidation [93]. Under these conditions, RER values below 0.7 from ketone production would result in an underestimation of CHO oxidation [138]. The LCHF group in our study was in a state of nutritional ketosis (Figure 13B) and therefore they were metabolically similar to those in the Phinney study [126]. However, the exercise intensity in our study was higher than that of Phinney et al. making a direct comparison difficult. Regardless, in contrast to Phinney et al. and in agreement with Lambert et al. we found a surprisingly high rate of CHO oxidation after adaptation to a LCHF diet at 72% of VO_{2max}.

Insulin plays a major regulatory role in CHO metabolism and generally acts to reduce EGP and promote the uptake of CHO into cells for subsequent oxidation or storage. The mixed diet group had significantly higher serum insulin concentrations than the LCHF group during exercise (Figure 13D). However, despite the finding of a statistical difference, the absolute difference was likely too small to account for the large differences in CHO metabolism. In other words, the high rates of CHO oxidation in the mixed diet group are unlikely to be due to a 1.5 U/l difference in insulin. Neither can the differences in EGP between groups be explained by these small differences in serum insulin concentration. In fact, the serum insulin concentration was slightly higher in the mixed diet group, despite significantly higher rates of EGP, indicating a mild hepatic insulin resistance during exercise after a mixed diet. Due to time and financial constraints, we did not measure plasma glucagon and catecholamines concentrations and there may have been differences in these hormones that could explain the differences in substrate oxidation rates. These metabolites will be measured in due course.

That CHO intake up-regulates CHO oxidation at rest and during exercise is well established [61, 122]. Therefore, the higher rates of CHO oxidation in the mixed diet group are likely due to the greater CHO intake and higher glycogen content after the mixed diet [8]. During the final 30 min of exercise, glucose Rd was 6.4 ± 1.0 mg/kg/min in the LCHF group and 8.2 ± 1.0 mg/kg/min in the mixed diet group. Our results provide support that the rate of CHO oxidation, via Rd glucose, directly regulates the rate of EGP [11, 139]. It has been proposed that glycogenolysis, in particular, is under the direct

control of plasma glucose concentrations [77, 144], which agrees with our finding that EGP is primarily modulated via GLY. This would explain how EGP was able to respond to Rd glucose in each group without major differences in serum insulin concentrations. It is therefore possible that the slightly higher serum insulin concentration in the mixed diet group is a result of the slightly higher need for glucose uptake from the circulation in the mixed diet group.

Despite the very different rates of CHO oxidation between groups, we found no difference in plasma lactate concentrations at any time-points during the 2 h ride (Figure 13C). Catabolism of muscle glycogen is considered the primary source of plasma lactate [57] and previous research has shown that reduced CHO availability is associated with reduced plasma lactate concentrations [8, 10, 74, 100]. However, the average plasma lactate concentrations during the 2 h ride was 3.2 ± 1.0 mmol/l in the LCHF group which was similar to the 2.8 ± 1.2 mmol/l in the mixed diet group. A possible reason for this is that the rate of glycolysis without subsequent glucose oxidation may be similar in both groups despite the higher glucose oxidation rate in the mixed diet group. Alternatively, lactate production and utilisation may be lower in the LCHF group than the mixed diet group, such that plasma lactate concentrations are similar but lactate flux is lower in the LCHF group.

Plasma lactate availability has been shown to regulate the rate of GNG during exercise [46, 133]. In the present study, any regulation of GNG by the plasma lactate concentration would likely have been similar in both groups. In other words, the rate of GNG may have been similar in the LCHF and mixed diet groups because the plasma availability of the main precursor for GNG was similar. Alternatively, the intensity of our exercise trial may have limited the rate of GNG. Results from Emhoff et al. [46] suggested that at 68% VO_{2max}, lactate availability may have limited GNG, whilst at lactate threshold (75% VO_{2max}), hepatic blood flow may have been the limiting factor [46]. Since our exercise trial was at approximately 72% VO_{2max}, this intensity may have been sufficient to reduce hepatic blood flow [112, 160] to the point that it was a limiting factor responsible for the finding that GNG was similar in the mixed diet and LCHF groups.

4.3) Fat oxidation and GNG regulation. We found that the rate of fat oxidation was significantly higher in the LCHF group than in the mixed diet group. The average fat oxidation rate during the 2 h ride was estimated at 1.2 ± 0.2 g/min in the LCHF group compared to 0.5 ± 0.2 g/min in the mixed diet group (Figure 5C). Even though there was a gradual increase in fat oxidation towards the end of exercise in the mixed diet group, their rate of fat oxidation during the final 30 min when GNG was quantified was 0.6 ± 0.2 g/min. Therefore, LCHF athletes had a fat oxidation rate double that of athletes who ate a mixed diet, during a period of exercise when the rate of GNG was not different

between groups. The rate of fat oxidation therefore does not appear to have any effect on the rate of GNG during exercise.

Despite the large group differences in fat oxidation rates, serum FFA concentrations were similar throughout exercise in LCHF and mixed diet athletes (Figure 13A). Although there was a gradual increase in serum FFA concentrations over time that occurred similarly in both groups, we found no association between fat oxidation rates and serum FFA concentrations. The concentration of FFA has previously been linked to a stimulation of GNG [13, 26, 30] but any regulatory effect of serum FFA concentrations on GNG in this study would have been the same in both groups. Similar FFA concentrations in fat-adapted and control diet athletes have been reported previously during exercise after an overnight fast [53, 126]. However, a CHO-rich meal prior to exercise reduces serum FFA concentrations during exercise whereas a LCHF meal does not change FFA concentrations compared to fasting [161]. Athletes in the mixed diet group in this study may have had higher serum FFA concentrations than they would usually have had after a pre-exercise CHO-containing meal or snack. Whether CHO-rich snacks and meals would inhibit GNG during exercise via a reduction in serum FFA concentrations invites further investigation.

Elevated plasma glycerol concentrations without a concomitant increase in plasma FFA concentrations is thought to occur when there are high rates of IMTG-derived FFA oxidation [86, 160]. We did not measure plasma glycerol concentrations but it is possible that these were elevated in the LCHF group compared to the mixed diet group. This is supported by the finding that LCHF diets have been associated with increased pre-exercise levels and utilisation of IMTG [66, 67, 164]. If there was increased glycerol availability in the LCHF group, this did not result in a higher rate of GNG compared to the mixed diet group. However, glycerol may have contributed to a greater extent towards glucose production than lactate in the LCHF group. Goedecke et al. demonstrated that differences in serum glycerol concentrations are more pronounced after five days of adaptation to a LCHF diet than after 15 days [53]. It is not known whether there is a difference during exercise in plasma glycerol concentration after long-term adaptation to a LCHF diet compared to a control diet.

Consistent with the high rates of fat oxidation in the LCHF group, these athletes were in a state of nutritional ketosis (Figure 13B). We found significantly higher levels of blood βHB at all time-points during the 2 h ride in the LCHF athletes compared to the mixed diet group. βHB was the only substrate, of those that we measured, that was significantly different between groups. Although ketones have been proposed to exert a regulatory effect on GNG [55, 140], they would not likely have played an important role in regulating the total rate of GNG during exercise in the current

study since GNG was the same in both groups. It is however unclear whether ketones influenced which precursors were used for GNG.

4.4) Summary of GNG and EGP regulation. Athletes adapted to long-term LCHF and mixed diets had large differences in whole body CHO and fat oxidation rates. However, these large differences in substrate utilisation were not associated with differences in serum FFA, serum insulin, plasma lactate or plasma glucose concentrations. Apart from βHB, the precursor, substrate and insulin concentrations were remarkably similar in both groups; such that it would have been difficult to distinguish groups solely on the basis of these measures. A LCHF diet has been shown to induce changes in plasma FFA and lactate concentrations after short-term adaptation [66] but our results suggest that after long-term adaptation these differences are less apparent. However, plasma substrate concentrations do not necessarily reflect the rates of substrate turnover and there could be differences in plasma substrate and/or gluconeogenic precursor turnover rates, even though plasma concentrations were not different (as is the case with plasma glucose).

Given the similarities in substrate and precursor concentrations, it is perhaps not so surprising that there was no difference in the rate of GNG in athletes adapted to long-term LCHF and mixed diets. However, rates of fat oxidation, CHO oxidation and blood ketone concentrations were very different between groups and were therefore not associated with the rate of GNG. Whether adaptation to a LCHF diet alters the contribution of the different precursors towards GNG requires further research. The higher rate of EGP in the mixed diet group was most likely the result of higher glucose Rd and not the result of hormonal regulation via insulin.

4.5) Muscle glycogen. Muscle glycogen content is likely an important component of endurance exercise performance, especially at moderate to high exercise intensities lasting 90 to 120 min [134, 156]. We found that pre-exercise muscle glycogen content was approximately 1.7 times lower in the LCHF group than in the mixed diet group (Figure 12). However, after 2 h of cycling, muscle glycogen content was similar in both groups at 42 ± 15 mmol/kg ww in the LCHF group and 47 ± 31 mmol/kg ww in the mixed diet group. A critically low level of muscle glycogen associated with the termination of exercise is around 25 mmol/kg ww [64], although other factors play a role in fatigue [115] and exercise is often terminated with muscle glycogen content higher than this “critical” limit [93, 126]. We measured EGP and GNG towards the end of submaximal exercise, at a time when muscle

glycogen, and presumably liver glycogen (since exercise occurred in the fasted state), may have been similar in both groups.

As is typically observed in fasted athletes adapted to CHO-rich diets [6], we found that RER decreased and fat oxidation increased throughout exercise in the mixed diet group. Towards the end of exercise, the mixed diet group would have been close to their maximum potential rate of fat oxidation [1]. At this stage, the rate of CHO oxidation was 2.3 times higher in the mixed diet group than the LCHF group. Our findings support the view that fat-adaptation induces a completely distinctive metabolic state which extends beyond the initial availability of muscle and liver glycogen [93, 126]. Even as CHO stores become similar, athletes eating CHO-rich diets are not able to increase fat oxidation to comparable rates of LCHF diet athletes, and therefore remain heavily reliant on CHO oxidation. The complex regulation of enzyme activities involved in fat and CHO oxidation may limit the potential for rapid adaptation to low carbohydrate availability [61].

Because of the high CHO oxidation rate of the mixed diet group, EGP was sufficient to meet only 20% of CHO oxidation requirements during the final 30 min of exercise while the rate of GNG was sufficient to meet only 7% of CHO oxidation needs (Table 6). Therefore, assuming the remainder of CHO was supplied from muscle glycogen, a muscle glycogenolysis rate of 2.31 g/min would have been required to maintain the workload during the final 30 min of exercise. By contrast the LCHF group was able to meet 42% of CHO oxidation requirements from EGP, even though EGP was significantly lower in the LCHF group than in the mixed diet group. The absolute rate of GNG was the same in the LCHF group and mixed diet group but because of the lower CHO oxidation rate in the LCHF group, 20% of the total CHO oxidation requirement was met through GNG alone. A rate of muscle glycogenolysis of only 0.75 g/min would have been required to meet the remainder of the CHO oxidation needs in the LCHF group. Theoretically, at this stage of a fasted ride when glycogen stores are equal between groups, the LCHF group would have a metabolic advantage over the mixed diet group because they can obtain more energy from fat and are not reliant on an exogenous supply of CHO to continue exercising.

Lambert et al. [93] inadvertently designed a study which tested this exact situation. Athletes adapted to either a LCHF or high-CHO diet were asked to cycle to exhaustion at 50% VO_{2max} immediately after an exhaustive high-intensity exercise bout. Glycogen was significantly reduced in both groups after the high-intensity bout and was 32 mmol/kg/min in the LCHF group and 73 mmol/kg/min in the high-CHO group. Despite lower starting glycogen, the LCHF group significantly outperformed the high-CHO group at the subsequent 50% VO_{2max} trial, cycling for 80 min compared to 43 min in the high-CHO group [93]. Rowlands et al. [135] reported a similar finding, that subjects

on a LCHF diet could sustain higher power outputs during a 100 km time-trial, which was performed immediately after a 45 min incremental exercise test.

Any performance benefits of long-term fat-adaptation would be most apparent at the stage during exercise when muscle glycogen content becomes similar to that of their competitors on higher CHO diets. In the present study this occurred at some point within 120 min of fasted sub-maximal exercise at 72% VO_{2max}. This point would also have been reached during any exercise bout in which end-exercise glycogen content is similar between LCHF, high-CHO and/or mixed diet athletes. Additionally, although our participants rode after an overnight fast, the convergence of muscle glycogen content would likely occur regardless of whether or not there was a pre-exercise meal/snack or CHO was ingested during exercise [80]. Ultra-endurance exercise is the most obvious situation in which athletes are required to exercise with low levels of muscle glycogen. However, significant reductions in muscle glycogen can also occur rapidly (10 to 30 min) [87, 93] and therefore the fat-adapted metabolic state may have benefits even during shorter endurance events such as marathon races.

4.6) EPG, GNG, CHO oxidation and exercise performance. The greatest limitation of a CHO-rich diet is the body's inability to reduce CHO oxidation requirements to match the progressive and inevitable reduction in endogenous CHO stores that occurs during exercise. Dietary CHO up-regulates CHO oxidation to such an extent that the contribution of GNG becomes very minor relative to CHO requirements. Therefore, the major benefit of fat-adaptation is to lower CHO oxidation rates to those that are more sustainable for GNG and EGP. The rate of GNG alone was sufficient to meet 20% of total CHO needs in the LCHF group, meaning that GNG made the same relative contribution to whole-body CHO oxidation needs as did the entire EGP in subjects in the mixed diet group. It is therefore not surprising that exogenous CHO and CHO-loading had such an impact on performance in previous studies using athletes adapted to high- and moderate-CHO diets.

To illustrate this point, if we ignored the contribution from muscle and liver glycogen to energy production, then the mixed diet group would have a shortfall of 2.7 g/min of CHO (Table 6). This shortfall of glucose has to be met by a combination of glycogen or exogenous sources. The LCHF group would have a shortfall of 1.0 g/min of CHO in the same situation. Glycogen and exogenous CHO therefore is of much greater importance in athletes on a mixed diet, and presumably is even more important in athletes on very high-CHO diets. Even if the mixed diet group were able to oxidise ingested CHO at a rate of 1.5 g/min, which is the highest rate that has been reported in the literature [76], they would still have a greater reliance on glycogen (1.1 g/min) than the LCHF group

without any oxidation of ingested glucose. However, since muscle glycogen is declining in the LCHF group, these athletes may benefit from the ingestion of CHO during exercise. An exogenous CHO oxidation rate of 1 g/min would be the absolute maximum required (at this exercise intensity) and an ingestion rate much lower than that would probably suffice since muscle and liver glycogen would make some contributions even in the closing stages of ultra-endurance events. Therefore, by reducing the need for exogenous CHO, the LCHF diet may be beneficial for athletes that experience gastrointestinal problems during exercise [28], especially if these problems relate to the ingestion of high quantities of CHO [43, 83].

Efforts to combine the two distinct metabolic states and raise muscle glycogen by CHO-restoration after fat-adaptation could therefore be counterproductive. If the lowering of CHO-oxidation requirements is the main benefit of a LCHF diet, then CHO-restoration would inhibit this adaptive process. By raising muscle glycogen content and up-regulating CHO-oxidation, the benefit of fat-adaptation is compromised. The rate of fat oxidation in all the CHO-restoration studies was lower than those of our habitual LCHF athletes [18, 19, 24, 60, 92, 135]. Additionally, many studies that did not involve CHO-restoration may not have had fat-adaptation because they only reduced habitual CHO intakes to between 10 and 30% of total calories and in some cases did not provide enough time for proper adaptation [53, 65, 67, 93, 135, 158]. Therefore, the exercise potential of true fat-adaptation, as first described by Phinney et al. [126] has not been properly studied in subsequent trials and clearly requires further investigation.

Nevertheless, there is still a common perception amongst most sport scientists that a LCHF diet will lead to a reduction in exercise performance, an increased perception of effort during exercise, an inability to perform high-intensity interval training and an inability to recover fast enough to maintain a heavy training load over successive days [20].

The theoretical disadvantage of fat-adaptation is that it limits the ability to perform high-intensity bursts of exercise, which are an important component of high-level endurance racing [20]. This is based on the concept that energy production from glycolytic pathways can occur at a much faster rate than from fat oxidation [63]. While theoretically plausible, it is not clear that substrate flux is a limiting factor for endurance exercise and the central regulation of muscle recruitment as the regulator must be considered [115]. An inability to perform high intensity exercise has yet to be demonstrated in practice. Results from high-intensity time to exhaustion [93], intermittent sprinting [60], Wingate testing [97], VO_{2max} testing [126] and strength testing [119] have shown that athletes on LCHF diets are capable of performing high-intensity exercise [64]. It has also been shown that LCHF athletes can utilise muscle glycogen at the same rate as high-CHO athletes during high-

intensity exercise [93]. In the maximal exercise test in our study, every individual in the LCHF group attained a RER value over 1.0 (1.07 ± 0.04) which is usually interpreted to mean that CHO is the primary fuel source.

The points mentioned above suggest that high glycolytic flux are possible. There is little doubt that CHO-adapted athletes are unable to attain very high rates of fat-oxidation [157] but there remains uncertainty over the high-intensity exercise capacity of athletes after prolonged fat-adaptation [20]. Further research into the capacity for high-intensity work after long-term fat-adaption is required before definitive answers can be provided [114].

4.7) Diet. By studying habitual LCHF athletes we were able to investigate endurance exercise physiology after long-term adaptation to a LCHF diet. The average length of time on the LCHF diet was 13 months and the shortest period was eight months. Since these participants had self-selected this diet, they were likely to have responded positively and had good compliance to the diet. Exercise after a LCHF diet has previously been studied after a seven-week adaptation period [65] and the longest adaptation period in highly-trained athletes was four weeks [126]. Therefore, to our knowledge, there is no study yet published of such long-term adaptation to the LCHF diet.

The LCHF diet has only recently gained popularity in South Africa, therefore it was surprising to find no significant difference between groups in the length of time that participants had been on their respective diets. However, there was a much larger range in the mixed diet group of 9 - 360 months, compared to 8 - 24 months in the LCHF group (Table 2). Three participants in the mixed diet group reported being on their current habitual diet for nine months. One participant changed his diet and had been a vegetarian for the past nine months. The other two had consulted sports nutritionists and altered their diets accordingly, although the changes to their diets probably did not involve as great a shift of macronutrients as did the change to a LCHF diet. The remaining four participants in the mixed diet group reported that their habitual diet was unchanged for 10 years or more.

We noticed a clear difference between the ages of those that responded to the trial recruitment advertisements for the LCHF versus the high-CHO diet. In fact, none of the responders for the LCHF diet were younger than 29 years of age. In contrast, there were many HCLF responders between the ages of 19 and 24 years who were not enrolled in this study. Younger athletes may be less likely to experiment with LCHF nutritional strategies, either because they have minimal health concerns or because they are still developing a nutritional strategy. By contrast, more experienced cyclists have

had time to assess their performance on high-CHO diets and may have been unhappy for performance or health reasons.

The average diet of our LCHF group consisted of 50 ± 20 g/d of carbohydrate (7% of calories), 231 ± 21 g/d of fat (72% of calories) and 147 ± 35 g/d of protein (21% of calories) (Table 2). This diet is higher in CHO and lower in fat than the diet used by Phinney et al. [126], which contained 85% of calories as fat and 15% as protein. However, it was low enough in CHO that participants on the LCHF diet were in a state of nutritional ketosis at rest and during exercise. Nutritional ketosis was achieved by these individuals in a free-living environment over a long-term period. They were also well-trained competitive cyclists and highly physically active. Our study therefore provides some evidence that a LCHF ketogenic diet is sustainable in practice in the long term. The diet is also unlikely to compromise the ability of individuals wishing only to perform moderate physical activity, in line with current physical activity guidelines [58].

The mixed diet consisted of 394 ± 102 g/d of CHO (51% of calories), 120 ± 52 g/d of fat (33% of calories) and 131 ± 51 g/d of protein (16% of calories) (Table 2). It has been reported previously that the nutrient composition of endurance trained athletes seldom has CHO intake in excess of 55% of total caloric intake [59, 62]. Our results provide further evidence that in practice a truly high-CHO diet is seldom strictly followed. Competitive endurance athletes are often advised to eat in the region of 8 - 10 g/kg/d of CHO [38] yet participants in our trial ate only 5.5 g/kg/d. Even then, this level of CHO inhibited fat oxidation rates compared to the LCHF group. A high-CHO group eating 8 - 10 g/kg/d of CHO may have had even lower fat oxidation rates and a greater dependence on CHO during exercise.

Protein intake was similar in both groups and was 1.9 ± 0.5 g/kg/d in the LCHF group and 1.8 ± 0.8 g/kg/d in the mixed diet group. The daily intake of protein in both groups is greater than the commonly recommended daily allowances (RDA) for protein [123]. However, this level of protein intake is safe in athletes [127], and the RDA for protein intake in endurance athletes may not accurately reflect the protein requirements of these athletes [124]. A key feature of the LCHF diet is to replace CHO calories with fat rather than protein. Despite this, LCHF diets are often mistakenly referred to as “high-protein diets”, which is often the source of criticism.

Sugar consumption in the mixed diet group was approximately seven times greater than that of the LCHF group (173 ± 60 g/d on the mixed diet versus 25 ± 15 g/d on the LCHF diet). One of the main sources of CHO in the mixed diet group were whole-grain products (Table 4), yet they still had a sugar intake equivalent to over 40 teaspoons of sugar per day. This may be of concern since excess

sugar intake, independent of calories, has been associated with an increased risk of metabolic disease [9].

PUFA consumption was similar in both groups despite the LCHF group eating approximately double the amount of fat of the mixed diet group. Generally, advocates of LCHF diets advise against the consumption of omega 6 fatty acids while conventional advice encourages the consumption of PUFA rather than saturated fat [71, 76]. The similarity in PUFA consumption between groups may be a reflection of this advice. In contrast, LCHF athletes ate more than double the amount of saturated fat and approximately double the amount of MUFA of the mixed diet group which also reflects dietary advice given by advocates of the LCHF diet.

4.8) Glucose tolerance. There were significant differences in oral glucose tolerance between the LCHF and mixed diet groups. Basal plasma glucose concentrations were similar in both groups prior to glucose ingestion but were higher in the LCHF group throughout the two-hour test (Figure 14A). Serum insulin concentrations were also similar in both groups at baseline. Insulin peaked later in the LCHF group and only returned to concentrations similar to baseline after 120 min in the LCHF group, compared to after 60 min in the mixed diet group (Figure 14B). Despite the LCHF group performing worse in the OGTT, six of the seven individuals in the LCHF group had normal glucose tolerance. One participant had a plasma glucose concentration of 8.14 mmol/l after 120 min and therefore met the criteria for impaired glucose tolerance. However, no individuals in either the LCHF group or the mixed diet group were insulin resistant or had impaired glucose tolerance according to HOMA-IR or the Matsuda indices, although the LCHF group had significantly worse insulin sensitivity according to the Matsuda index (Figure 15).

Previous experiments have shown that high-CHO diets improve insulin mediated glucose disposal [15, 91]. Additionally, after a LCHF diet, there is reduced insulin-stimulated oxidative glucose disposal and high resistance to insulin-stimulated decreases in rates of fat oxidation [11, 41]. Resistance to the insulin-stimulated suppression of EGP after LCHF diets has also previously been demonstrated [11]. Therefore a high-fat diet is thought by many to induce insulin resistance and play a role in the development of chronic diseases related to insulin resistance.

However, rather than inducing a pathological state of insulin resistance and glucose intolerance, a more likely scenario is that insulin action is altered once in a fat-adapted metabolic state [11, 23, 91]. The LCHF group in our study probably relied on fat-oxidation during the OGTT, with the majority of glucose disposal resulting in glycogen storage [11, 41]. By contrast the rapid disposal of glucose in

the mixed diet group may have been due to high rates of whole-body glucose oxidation in conjunction with glycogen storage [11, 41]. From our EGP results, it is clear that a long-term LCHF diet has not induced hepatic insulin resistance, since the rates of EGP are lower at rest and during exercise in the LCHF group than in the mixed diet group, despite similar plasma glucose and serum insulin concentrations. Rather, it is the mixed diet group that has a mild hepatic insulin resistance relative to the LCHF group. The appearance of better insulin sensitivity and glucose tolerance after a high-CHO or mixed diet is created by the high rates of whole-body CHO oxidation that occur after high dietary CHO intakes [11].

In fact, the LCHF diet is particularly effective in managing conditions associated with insulin resistance [48, 120] and the value of an OGTT for the diagnosis of glucose tolerance and insulin resistance is questionable in a population that would rarely, if ever, eat 75 g of glucose in a single day, let alone in a single meal. We found evidence of impaired glucose tolerance in the LCHF group according to the OGTT and Matsuda index. However, our results should be interpreted with caution as the OGTT may not be a valid test for metabolic health in individuals on a LCHF diet.

4.9) Limitations. For methodological purposes, participants were required to fast overnight prior to the tracer infusion trial for the measurement of EGP and fractional GNG. Athletes on habitual high-CHO and mixed diets would be unlikely to compete and train in the fasted state. Therefore, we have not studied the mixed diet group in a state which most accurately represents their habitual practices.

We used indirect calorimetry and stoichiometric equations to estimate substrate utilisation. A limitation of this method is that RER is the sum of all the metabolic processes in the body and therefore the reported CHO and fat oxidation rates are not directly measured but rather estimates based on O_2 utilisation and CO_2 production. There are many factors in the present study which could influence the reported rates of CHO and fat oxidation [50]. Perhaps the most pertinent factor relating to our study is the potential for the production of ketones without subsequent oxidation [138].

Habitual diets in this study were estimated based on self-reports by the participants. While we are reasonably certain that the three day diet logbooks are a good reflection of dietary intake for the period preceding the testing visit, we cannot be certain that these individuals had truthfully followed a strict LCHF diet, or mixed diet, for the entire period that they specified. However, we used a non-quantitative food frequency questionnaire and interviewed the participants extensively to learn

about their dietary habits and knowledge of nutrition. We also did not control dietary intake on the evening before the trial (we did not provide meals). This was done intentionally to reflect that participant's chosen diet but it may have caused some additional variance in the results.

By testing habitual LCHF participants, there may have been some bias towards the selection of individuals that had responded positively to the diet. There may be individuals who respond poorly to the LCHF diet and these individuals would not have been tested.

4.10) Summary and conclusions. We studied substrate utilisation and the rates of EGP, GLY and GNG during exercise in seven male cyclists who habitually ate a LCHF diet, and seven control cyclists who habitually ate a mixed diet. To our knowledge, this is the first time that total GNG from all precursors has been quantified during exercise in well-trained athletes and the first time that EGP, GNG and GLY have been quantified during exercise in athletes on LCHF diets.

While there were profound differences in fat and CHO oxidation rates between groups, we found no difference in the rates of GNG between the LCHF and mixed diet groups, either at rest or during exercise. However, the rates of EGP under both conditions were greater in the mixed diet group than the LCHF group, due to higher rates of GLY. We conclude that rates of GNG are relatively stable across a broad range of habitual diets that can significantly alter substrate utilisation, and that dietary CHO modulates the rates of EGP via alterations in rates of GLY, both at rest and during exercise.

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APPENDIX 1

American Heart Association (AHA) / American College of Sports Medicine ACSM) Health/Fitness Facility Preparticipation Screening Questionnaire*

Asses your health needs by marking all *true* statements

History

You have had:

__ a heart attack
__ heart surgery
__ cardiac catheterisation
__ coronary angioplasty (PTCA)
__ pacemaker/implantable cardiac defibrillator/rhythm disturbance
__ heart valve disease
__ heart failure
__ heart transplantation
__ congenital heart disease

Symptoms

__ You experience chest discomfort with exertion
__ You experience unreasonable breathlessness
__ You experience dizziness, fainting, or blackouts
__ You take heart medications.

Other health issues

__ You have diabetes.
__ You have asthma or other lung disease.
__ You have burning or cramping sensation in your lower legs when walking short distances.
__ You have musculoskeletal problems that limit your physical activity
__ You have concerns about the safety of exercise.
__ You take prescription medication(s).
__ You are pregnant.

If you marked any of the statements in this section, consult your physician or other appropriate health care provider before engaging in exercise. You may need to use a facility with a ***medically qualified staff***

Cardiovascular Risk Factors

__ You are a man older than 45 years.
__ You are a woman older than 55 years, have had a hysterectomy, or are postmenopausal.
__ You smoke, or quit smoking within the previous 6 months.
__ Your blood pressure is > 140/90 mm Hg.
__ You do not know your blood pressure
__ You take blood pressure medication.
__ Your blood cholesterol level is > 200 mg/dL.
__ You do not know your cholesterol level
__ You have a close blood relative who had a heart attack or heart surgery before age 55 (father or brother) or age 65 (mother or sister).
__ You are physically inactive (i.e., you get < 30 minutes of physical activity on at least 3 days per week)
__ You are > 20 pounds (9.07185 kg) overweight.

If you marked two or more of the statements in this section, you should consult your physician or other appropriate healthcare provider before engaging in exercise. You might benefit by using a facility with a professionally qualified exercise staff to guide your exercise programme.

__ None of the above is true.

You should be able to exercise safely without consulting your physician or other appropriate health care provider in a self-guided programme or almost any facility that meets your exercise programme needs.

Participant name:	
Signature	Date

APPENDIX 2

CRD1: Medical, fitness and diet questionnaire

PERSONAL DETAILS

Name: _____

Surname: _____

Postal address: _____

_____ Code: _____

Email address: _____

Phone number: _____ Cell phone: _____

Date of birth: _____ Age: _____

Gender: _____ Occupation: _____

Race (Optional): _____

EMERGENCY CONTACT DETAILS

Name and surname _____ Relation _____

Home phone _____ Cell phone _____

Usual doctor _____ Telephone _____

CURRENT CYCLING ABILITY

1. How many years have you been cycling regularly for? (tick)

0-1 years	
1-2 years	
2-4 years	
More than 4 years	

2. Over the past 3 months, on average**,** how many hours have you cycled per week? (tick)

0-4 hours	
4-7 hours	
7-10 hours	
10-13 hours	
> 13 hours	

3. Over the past 3 months, have you performed any of the following cycling sessions? (Y/N)

Long slow distance		Sprints	
High intensity intervals		Time trials	
Hill training		High cadence training	

4. How would you describe your current cycling? (tick)

I cycle occasionally for fun and fitness	
I cycle regularly but don't take it that seriously and don't enter races	
I cycle regularly and enter races but don't like to push myself	
I train hard and race to do as well as I can	
I am a professional cyclist	

5. Do you own or have access to a bicycle in good working order that you can use for the trial? Yes _____ No _____ Road/MTB _____ _____

6. Have you been training and/or racing on a bicycle consistently in the past 3 months? If not, please explain. _____ _____

7. How would you describe your current cycling fitness compared to your best level of fitness?

Very unfit	
Reasonably fit	
Fit	
Very fit	
The most fit I have ever been	

8. In the past **3 months**, how many times have you ridden continuously for more than 2 hours in one session at a relatively intense pace?

None	
1-3 times	
3-6 times	
More than 6 times	

9. Please fill in the details of 3 cycle races, which you have completed within the past 6 months. If possible, choose popular races, which reflect your best performances.

Date	Race name	Dist.	Road/MTB	Time	Pos.

10. Do you think you can currently ride at 55% of your peak power output for 2 hours? This would be at approximately 85% of maximal effort or roughly 85% of maximal heart rate, for 2 hours? In other words, it would be equivalent to a hard 2-hour training ride.

I don't understand the intensities you described?	
No I can't.	
I am not sure.	
Yes I think so.	
Yes I can.	

RECENT INJURIES

Have you suffered from any serious sports related pain or injuries in the past 2 months?

Yes _____ No _____

If yes, please provide details:_____

_____

If yes, do they interfere with your cycling ability?

Yes _____ No _____

If yes, please provide details:_____

_____

MEDICATION AND SUPPLEMENT USE

What medication, if any, are you currently using?

Name of medication For what condition? Period taken

_____

_____

_____

_____

_____

What chronic medication, if any, have you taken during the past three months, but are no longer using?

Name of medication For what condition? Period taken

_____

_____

_____

_____

_____

What dietary supplements / vitamins, if any, are you currently using?

Type of supplement Name For what condition? Period taken

SMOKING

Do you **currently smoke** any tobacco products, such as cigarettes, cigars, or pipes (tick)?

Yes _____ No _____ If yes, for how long _____

If not, have you smoked any tobacco products in the past year (tick)?

Yes _____ No _____ If yes, for how long _____

How many cigarettes do/did you smoke per day (tick)?

<10/day _____ 10-20/day _____ 20-30/day _____ >30/day _____

ALCOHOL INTAKE

Note: 1 standard alcohol unit is equal to 10 ml of pure alcohol:

~ 200 ml of beer (a can of beer is 1 and a half units)

~100 ml glass of wine (a small glass is 1 unit)

~1 tot (25 ml) spirits is 1 unit

1. On a typical **WEEK day**, how many standard alcohol units do you drink?

0	
1 or 2	
3 or 4	
5 or 6	
7 to 9	
10 or more	

2. On a typical **WEEKEND**, how many standard alcohol units do you drink?

0	
1 or 2	

3 or 4	
5 or 6	
7 to 9	
10 or more	

DIET

1. Do you follow a high carbohydrate / low fat diet or a high fat / low carbohydrate diet?

   High Carb _____ Low Carb _____

   Please briefly describe your eating plan.

   _____

   _____

   _____

   How long (in years / months) have you followed your current way of eating? _____

   Why did you decide to follow this eating plan?

   _____

   _____

   _____

2. Have you started a new eating plan or significantly changed the **type** of foods that you typically eat, **within the last 6 weeks**? Yes _____ No _____

   If yes, please describe what changes you have made:_____

   _____

   _____

   _____If yes, what were the reasons that you changed your diet:

   _____

   _____

   _____

3. Have you significantly changed the **amount** of food that you typically eat, in the last 6

weeks?

Yes _____ No _____

If yes, please describe what changes you have made:_____

_____

_____

_____

4. What is your current weight (roughly)? _____

5. What is your height (roughly)? _____

6. In the **past 6 weeks** has your body weight changed by more than 3kg at any stage?
   Yes _____ No _____ I don't know _____
   If yes, what was your lowest and highest weight that you measured during this time?
   Lowest_____Highest_____

7. In the **past 6 months** has your body weight changed by more than 3kg at any stage?
   Yes _____ No _____ I don't know _____
   If yes, what was your lowest and highest weight that you measured during this time?
   Lowest_____Highest_____

8. In **the past year** has your body weight changed by more that 5kg at any stage?
   Yes _____ No _____ I don't know _____
   If yes, what was your lowest and highest weight that you measured during this time?
   Lowest_____Highest_____

FOOD AND DRINK DURING EXERCISE

1. Which foods and drinks do you usually consume during your training rides or races?

Before: _____

_____

During: _____

_____

After: _____

_____

APPENDIX 3

Food and drink		**Serving size**	**0**	**1 or less per month**	**2-3 per month**	**1-2 per week**	**3-4 per week**	**5-6 per week**	**1 per day**	**2-3 per day**	**More than 3 per day**
Fruit juice		1 glass/ 300ml									
Fizzy soft drink e.g. Coke, Fanta etc.	Diet	1 can/ 340 ml									
	Regular	1 can/ 340 ml									
Sports energy drink	Low carb	1 glass/300 ml									
	Regular	1 glass/300 ml									
Sports energy bars	Low carb	1 bar ~ 50g									
	Regular	1 bar ~ 50g									
Beer	Regular	1 can/ 340 ml									
	Low calorie	1 can/ 340 ml									
	Low alcohol	1 can/ 340 ml									
Wine		1 glass/ 200ml									
Alcoholic coolers		1 bottle/250ml									
Bread	White	1 slice/ half roll									
	Brown	1 slice/ half roll									
	Whole wheat	1 slice/ half roll									
	Rye	1 slice/ half roll									
	Other	1 slice/ half roll									
Crackers/ crisp breads/ savory biscuits		3 biscuits									
Spread (e.g. on bread/ crackers)	Butter	1 table spoon									
	Margarine	1 table spoon									
	Jam	1 table spoon									
	Peanut butter	1 table spoon									
	Other	1 table spoon									
Milk (including in tea, coffee, flavored milk, cereal)	Full cream	1 glass/ 300ml									
	Low fat	1 glass/ 300ml									
	Fat free	1 glass/ 300ml									
	Soya milk	1 glass/ 300ml									
Cream (e.g. in coffee)		2 table spoons									
Yoghurt	Fat free/ low fat plain	1 cup/ 250ml									
	Low fat flavored	1 cup/ 250ml									
	Full fat/Double cream	1 cup/ 250ml									
Cheese		1 bread sized slice									
Sugar added to drinks, cereal etc.		1 tea spoon									
Cereal: Main types: .		1 bowl									

Food and drink		**Serving size**	**0**	**1 or less per month**	**2-3 per month**	**1-2 per week**	**3-4 per week**	**5-6 per week**	**1 per day**	**2-3 per day**	**More than 3 per day**
Porridge/ oats		1 bowl									
Pasta/ noodles including lasagne		2 cups cooked									
Pizza		1 medium									
Pies		1 pie									
Fast food		1 meal									
Rice		1 cup cooked									
Quinua		1 cup cooked									
Lentils		1 cup cooked									
Cous cous		1 cup cooked									
Barley		1 cup cooked									
Kidney beans		1 cup									
Baked beans		1 cup									
Potatoes (including french fries)		1 cup									
Other starchy veg (Sweet potato, butternut, pumpkin, carrots, beetroot, squash, corn, turnips)		1 cup cooked									
Other non starchy vegetables e.g Broccoli, tomato, Lettuce, spinach, chard, cabbage, cauliflower, green beans, celery, cucumber, mushrooms, olives, onion, peppers		1 cup									
Salad dressing	Low fat	1 table spoon									
	Normal	1 table spoon									
Oils eg olive, coconut, sunflower		1 table spoon									
Banana, pear, apple, citrus, grapes		1 med fruit									
Berries, melon		half cup									
Avocado		Half fruit									
Other fresh fruit (excluding above)		1 med fruit/ half cup									
Dried fruit (including raisins)		Half cup									
Peanuts, cashews,		Small hand full									
Macadamia nuts		Small hand full									
Other nuts (excluding above)		Small hand full									
Meal replacement shakes What type:_____		1 glass/ 300ml									
Fish	Dark meat fish e.g. salmon, tuna, sardines, mackerel, trout, swordfish, snoek	1 dinner portion									
	Light meat fish e.g. hake, haddock	1 dinner portion									
	How many times was the fish covered in bread										

	crumbs or batter										
Food and drink		**Serving size**	**0**	**1 or less per month**	**2-3 per month**	**1-2 per week**	**3-4 per week**	**5-6 per week**	**1 per day**	**2-3 per day**	**More than 3 per day**
Fresh red meat (beef, lamb, pork, ostrich, etc., excl precessed meats)	Lean/ fat trimmed off	1 dinner portion/ 200 g									
	Including fat	1 dinner portion/ 200 g									
	How many times was the meat covered in bread crumbs										
Processed meats (salami, sandwich ham, chicken roll etc)		For example on a sandwich									
Poultry (chicken, turkey etc.)	Skin removed	1 piece									
	With Skin	1 piece									
Eggs		1 egg									
Chocolate		1 bar ~ 50g									
Sweets		1 packet ~ 50g									
Cakes, muffins, brownies, waffels		1 serving									
Pastries, croissants, etc.		1 croissant									
Sweet biscuits		1 buiscuit									
Dessert (ice cream, puddings etc, excluding baked goods above)		1 bowl									
Other food you eat more than once/ week: _____											
Other food you eat more than once/ week: _____											
Other food you eat more than once/ week: _____											
Other food you eat more than once/ week: _____											

Food Frequency Questionnaire – Tick how often you consumed foods in the past 6 weeks