Exercise-nutrient interactions: Effects on substrate metabolism and performance

Submitted by

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No other person's work has been used without due acknowledgment in the main text of the thesis. All research procedures reported in the thesis received the approval of the relevant Ethics/Safety Committees.

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"If your dreams don't scare you, they are too small" Richard Branson

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Contents

List of publications related to thesis

1. Hawley, J.A., & **Leckey, J.J**. (2015). Carbohydrate dependence during prolonged, intense exercise. *Sports Medicine, 45,* 5-12. doi: 10.1007/s40279-015-0400-1

2. **Leckey, JJ**, Burke LM, Morton JP & Hawley JA. (2015). Altering fatty acid availability does not impair prolonged, continuous running to fatigue: Evidence for carbohydrate dependence. *J Appl Physiol, 120(2),* 107-13. doi: 10.1152/japplphysiol.00855.2015

3. **Leckey JJ,** Ross ML, Quod M, Hawley JA, Burke LM. (2017) Ketone diester ingestion impairs time-trial performance in professional cyclists. *Front Physiol, 8*, 806. doi: 10.3389/fphys.2017.00806

4. **Leckey JJ**, Hoffman NJ, Devlin BL, Parr EB, Trewin, Stepto NK, Morton JP, Burke LM, Hawley JA. (2018) High dietary fat intake increases fat oxidation and reduces skeletal muscle mitochondrial respiration in trained humans. *FASEB J,* Jan 17. Doi: 10.1096/fj.201700993R

List of conference presentations

1. **Leckey, JJ**, Burke LM, Morton JP & Hawley JA. (2015). Altering fatty acid availability does not impair prolonged, continuous running to fatigue: Evidence for carbohydrate dependence. *J Appl Physiol*, *120(2),* 107-13.

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2. **Leckey, JJ**, Hoffman, NJ, Parr, EB, Devlin, BL, Trewin, AJ, Stepto, NK, Morton, JP, Burke, LM, Hawley, JA. (2016). Does low carbohydrate or high fat availability promote fat oxidation?

   Presented at Canadian Society of Exercise Physiology, Victoria, Canada, October 2016.

   [Oral presentation]

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Abstract

During prolonged (> 90 min), continuous steady-state exercise, skeletal muscle is fuelled by both carbohydrate (CHO) (i.e. muscle and liver glycogen, blood glucose and muscle, blood and liver lactate) and fat substrates (i.e. adipose and intramuscular triglycerides [IMTGs], blood-borne free fatty acids [FFAs] and TGs). The specific pattern of substrate oxidation is influenced by the relative exercise intensity, an individual's training status and their preceding diet. However, it is well accepted that when exercising at high relative intensities (i.e. > 70% maximal oxygen uptake [$\dot{V}O_2max$]), CHO-based fuels are the predominant fuel source. Despite CHO being important for sustaining prolonged exercise, recent attention has focused on exercise-nutrient protocols that reduce skeletal muscle dependence on CHO fuel sources and, instead, increase reliance on fat-based fuels. Such strategies include high-fat, low-CHO diets, training with low endogenous and exogenous CHO availability and oral ketone supplementation. In theory, strategies that "spare" the oxidation of CHO substrates should enhance endurance exercise performance. This thesis comprises a series of independent but related studies investigating the effects of manipulating both endogenous and exogenous fat availability on substrate metabolism, skeletal muscle adaptations and exercise performance.

Study 1 (described in chapter 4) investigated the effect of decreasing circulating FFA availability prior to and during half-marathon running. FFA availability was suppressed via the administration of nicotinic acid, ingested prior to and during exercise. The suppression of lipolysis and the exercise-induced rise in plasma FFAs did not impair half-marathon running capacity. When running at ~80% $\dot{V}O_2max$ for ~90 min there was a small but obligatory use of fat substrates, independent of CHO intake pre- and during exercise. However, CHO was the predominant fuel source, contributing between 80-90% to total energy expenditure.

Study 2 (described in chapter 5) examined the effects of ingesting a ketone diester on circulating ketone bodies, substrate metabolism and cycling performance under nutritional conditions replicating an elite professional cycling time-trial. Ketone ingestion increased circulating β-hydroxybutyrate and acetoacetate concentrations. Despite optimal nutritional support, the ketone diester was also associated with gut discomfort and an increased perception of effort, leading to an impairment of cycling time-trial performance.

Study 3 (described in chapter 6) manipulated endogenous fat and CHO availability via daily energy intake, to determine whether the metabolic perturbations from a high-fat diet are driven by high-fat or low-CHO availability. Participants consumed five days of a high-fat or high-protein diet (~65% energy intake), while 'clamping' CHO consumption to < 20% energy intake. When compared to an isoenergetic high-protein diet, five days' adaptation to a high-fat diet resulted in greater whole-body rates of fat oxidation during submaximal cycling and decreased skeletal muscle mitochondrial respiration supported by octanoylcarnitine and pyruvate as well as uncoupled respiration at rest. Following one day of a high-CHO diet mitochondrial respiration returned to pre-diet, however whole body rates of substrate oxidation were only partially rescued.

This series of research studies contributes new knowledge to the literature by demonstrating that 1) fat substrates contribute < 20% to energy expenditure during prolonged, high-intensity running, independent of pre-exercise CHO intake 2) ketone diester ingestion impairs cycling time trial performance and is associated with a higher perception of effort, despite optimal nutritional feeding and 3) high dietary fat rather than low-CHO intake contributes to reductions in mitochondrial respiration and increases in whole-body rates of fat oxidation following a high-fat, low-CHO diet. However, this reduction can be partially rescued

following one day of a high-CHO diet. This novel information provides evidence that high-fat diets and exogenous ketone drinks are not advantageous for an athletes training and competition due to their detrimental effects on substrate metabolism and skeletal muscle adaptations. Athletes should instead ensure high-CHO availability prior to and during competition to maximise whole-body rates of CHO oxidation rates.

List of Abbreviations and Nomenclature

100SS	100 minute steady state ride
AcAc	Acetoacetate
ACC	Acetyl CoA Carboxylase
ADP	Adenosine diphosphate
AMPK	AMP-activated protein kinase
ATP	Adenosine triphosphate
βHB	βeta-hydroxybutyrate
BIOPS	Biopsy preservation solution
BM	Body mass
CHO	Carbohydrate
CI	Mitochondrial Complex I
CII	Mitochondrial Complex II
CIII	Mitochondrial Complex III
CIV	Mitochondrial Complex IV
CV	Mitochondrial Complex V
CPT1	Carnitine palmitoyltransferase-1
EGTA	Ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EI	Energy intake
ES	Effect size
ETF	Electron-Transferring Flavoprotein

ETFp	Oxidative phosphorylation electron-transferring flavoprotein respiration
ETS	Electron Transport System
FAT/CD36	Fatty acid translocase /CD36
FFA	Free fatty acids
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
IMTG	Intramuscular triglycerides
LDL	Low density lipoprotein
MAP	Maximal aerobic power
mTOR	Mammalian target of rapamycin
NA	Nicotinic acid
OXPHOS	Oxidative phosphorylation
PDH	Pyruvate dehydrogenase
PPO	Peak power output
RER	Respiratory exchange ratio
RPE	Ratings of perceived exertion
RPS6	S6 Ribosomal protein
RQ	Respiratory quotient
SUIT	Substrate-uncoupler-inhibitor-titration
TCA	Tricarboxylic acid cycle
TT	Time-Trial
TG	Triglycerides
$\dot{V}CO_2$	Volume of carbon dioxide produced

$\dot{V}O_2$ Volume of oxygen consumed

$\dot{V}O_2max$ Maximal oxygen uptake

$\dot{V}O_2peak$ Peak oxygen uptake

1. Chapter 1- Introduction and overview

During prolonged (> 90 min), endurance based exercise, skeletal muscle is primarily reliant on oxidative metabolism, utilising both carbohydrate (CHO) and fat based fuels as substrates for muscle contraction (Figure 1.1) (Brooks & Mercier, 1994). The ability of the skeletal muscle to match adenosine triphosphate (ATP) production with ATP hydrolysis is reflective of the size of the available substrate pools and the muscles 'metabolic flexibility', defined as the ability to appropriately transition between substrates in response to hormonal and/or contractual stimuli (Impey et al., 2018; Kelley, 2005).
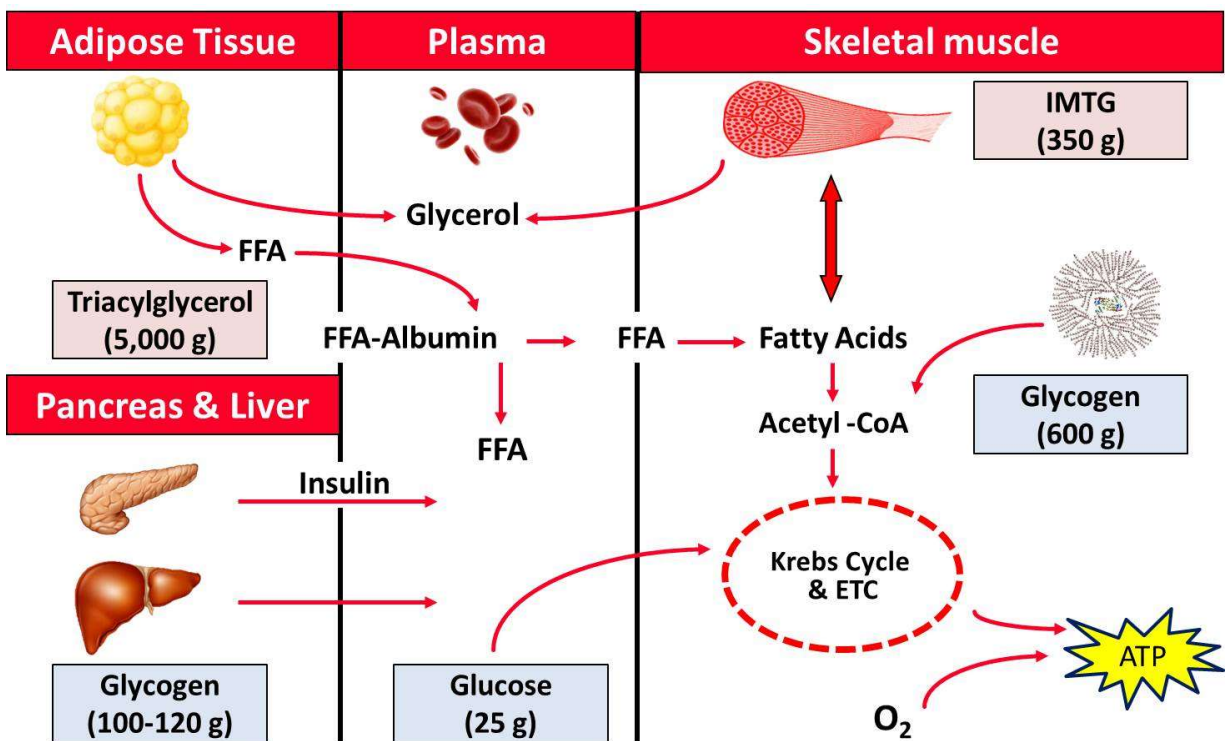
Figure 1.1. Endogenous fuel stores to support skeletal muscle contraction in trained endurance athletes. FFA, free fatty acids; IMTG, intramuscular triglycerides; ETC, electron transport chain; ATP, adenosine triphosphate. Adapted from Coyle, 1997.

The major metabolic consequences of the adaptations of skeletal muscle to endurance training are a slower utilisation of CHO-based fuels, a greater reliance on fat-based fuels and less lactate production during low- to moderate-intensity exercise (i.e., 45-65% of $\dot{V}O_2$max) undertaken at the same absolute (pre-training) exercise intensity (Bergman et al., 1999). These adaptations, in part, underpin the substantial increases in submaximal exercise capacity observed following endurance training (Holloszy, 1967). Accordingly, many athletes and coaches steadfastly believe that fat-based fuels play an important role while training for and competing in endurance events lasting up to 3 h and that training and/or nutritional strategies that "spare" CHO-based fuels and enhance the oxidation of fat-based fuels will improve exercise capacity.

However, rates of fat oxidation over a wide range of speeds/power outputs are not substantially altered after endurance training when exercise is undertaken at the same *relative* (post-training) intensity. This is because a major goal of training for performance enhancement is to promote skeletal muscle adaptations that allow an athlete to work at both higher absolute and relative power outputs/speeds becoming more rather than less reliant on CHO-based fuels. In support of this contention, competitive endurance athletes train and race at intensities that are highly dependent on CHO for muscle contraction and consume diets that are CHO-rich (Burke et al., 2001; O'Brien et al., 1993; Spriet, 2007; Torrens et al., 2016). Fat-rich diets do not "spare" CHO (i.e., muscle glycogen) or improve training capacity or performance, but rather alter skeletal muscle adaptations which impair the high rates of muscle glycogenolysis that are a necessary prerequisite for successful endurance performance (Stellingwerff et al., 2006). Exogenous ketone supplementation provides an alternative fuel to CHO and fat-based sources, although the effects of such supplementation on prolonged endurance performance or in

situations replicating real-world conditions are yet to be investigated.

This thesis aims to address several gaps in the literature and is comprised of three independent but related studies to determine 1) the effect of suppressing lipolysis on substrate selection and prolonged, high-intensity running capacity 2) the effects of exogenous ketone ester ingestion on substrate metabolism and performance and 3) the effects of increased dietary fat availability and a low-CHO intake on the mechanisms underpinning skeletal muscle adaptations. A review of the current literature can be found in the subsequent chapter and chapters four, five and six consist of the experimental studies listed below. The novel findings from the three studies and directions for future research are discussed in chapter seven.

1) Study 1: Altering fatty acid availability does not impair prolonged, continuous running to fatigue: Evidence for carbohydrate dependence
2) Study 2: Ketone diester ingestion impairs time-trial performance in professional cyclists
3) Study 3: High dietary fat intake increases fat oxidation and impairs skeletal muscle mitochondrial respiration in trained humans

2. Chapter 2- Literature review

Publication statement:

This chapter incorporates the review published in *Sports Medicine* (Hawley & Leckey, 2015). For the purpose of this thesis, sections have been updated with relevant research published after 2015.

Hawley, J.A., & **Leckey, J.J**. (2015). Carbohydrate dependence during prolonged, intense exercise. *Sports Medicine, 45,* 5-12. doi: 10.1007/s40279-015-0400-1

2.1 Effects of endurance training on patterns of substrate oxidation

It is widely accepted that after a period of endurance exercise training the non-protein respiratory exchange ratio (RER, volume of carbon dioxide produced/volume of oxygen consumed [$\dot{V}CO_2$/ $\dot{V}O_2$]) is lower, muscle glycogenolysis is less and fatty acid oxidation is greater compared to before training at the same absolute power output/speed (Bergman & Brooks, 1999; Bergman et al., 1999; Brooks & Mercier, 1994; Holloszy & Coyle, 1984). The phenomenon of increased fat utilisation and CHO "sparing" in response to endurance training was first observed in early investigations using solely RER measures as an indirect estimate of whole-body substrate use (Krogh & Lindhard, 1920). It was not until the reintroduction of the percutaneous needle biopsy technique into exercise physiology in the 1960s that direct evidence of a training-induced muscle glycogen sparing effect was verified in humans (Bergstrom et al., 1967; Karlsson et al., 1974).

In recent years indirect calorimetry in combination with isotopic tracer techniques and/or direct measures of substrate utilisation from serial biopsies have been used to evaluate the regulation of endogenous fat and CHO metabolism in relation to exercise of varying intensities and a number of nutritional interventions (Arkinstall et al., 2004; Bergman et al., 1999; Hawley et al., 1994; Romijn et al., 1993; van Loon et al., 2001). Using these approaches, a large body of experimental evidence supports the overall interpretation that endurance training reduces the amount of CHO-based fuels oxidised during submaximal exercise, while the contribution from fat-based fuels to total energy expenditure increases (Brooks & Mercier, 1994; Gollnick, 1985; Hawley et al., 2015; Holloszy & Coyle, 1984).

While these general conclusions are valid, several caveats need to be considered. First,

in the overwhelming majority of studies, subjects have only been tested at the same absolute (pre-training) work-rate. Thus, even after short-term (i.e., 2-12 wk) training interventions that typically improve $\dot{V}O_2max$ by 10-15%, the relative intensity of exercise (as a proportion of the new, higher $\dot{V}O_2max$) is usually 10% lower post-training. Not surprisingly, under these conditions, rates of fat oxidation are always higher. Remarkably, few studies have tested subjects at both the same absolute and relative exercise intensities after a period of endurance training. Secondly, most studies employ previously untrained, predominantly male subjects to investigate the effects of short-term training interventions on patterns of substrate use. While there are major physiological and metabolic changes induced by the implementation of endurance training regimens in previously sedentary subjects, the results from these investigations bear little relevance to well-trained athletes with a history of many years of training. Third, subjects are frequently tested at a single submaximal work-rate before and after a training intervention, mostly during exercise of low- or moderate-intensity (i.e., < 65% of $\dot{V}O_2max$) and seldom at higher work rates at which fat oxidation would be minimal (Achten & Jeukendrup, 2003; Romijn et al., 1993; van Loon et al., 2001). Finally, in the majority of studies, subjects are tested after a 10-12 h overnight fast. Notwithstanding the fact that competitive athletes are unlikely to commence the majority of training sessions and/or races with low-CHO availability, such conditions would be expected to increase the contribution of fat-based fuels to total energy requirements, at least during exercise of low- to moderate-intensity.

Experimental evidence to support the contention that prior endurance training has little effect on patterns of substrate utilisation and that both untrained and highly trained individuals are CHO-dependent at high exercise intensities comes from the work of Bergman and colleagues (Bergman & Brooks, 1999; Bergman et al., 1999). In one study, these workers

trained nine male subjects for nine weeks and measured whole-body RER, leg respiratory exchange quotient (RQ), tracer-derived measures of FFA fractional extraction and muscle TG utilisation during 1 h of cycling at two exercise intensities before (45 and 65% of $\dot{V}O_2$max) and after training (65% of pre-training $\dot{V}O_2$max, the same absolute intensity, and at 65% of post-training $\dot{V}O_{2max}$, the same relative intensity). The training programme was successful in promoting significant metabolic adaptations including a 15% increase in $\dot{V}O_2$max. When subjects were tested at the same absolute (pre-training) intensity (i.e., 65% of pre-training $\dot{V}O_2$max representing 54% of post-training $\dot{V}O_2$max), there were increases in rates of whole-body fat oxidation (a decrease in RER from 0.96 to 0.93). However, as represented in Figure 2.1, when subjects were tested at the same relative intensity (65% of pre- and post-training $\dot{V}O_2$max), RER values (0.95), leg RQ (0.98), net FFA uptake and muscle TG utilisation were not different. While the absolute amount of CHO- and fat-based fuels increased, there was only a small (3%), insignificant difference in CHO and fat utilisation from pre- to post-training at the same relative intensity. These data demonstrate that the balance of substrate utilisation is only slightly modified by prior endurance training and that CHO-derived energy sources are the major fuel source for working muscle even during one hour of moderate-intensity exercise (Bergman et al., 1999).
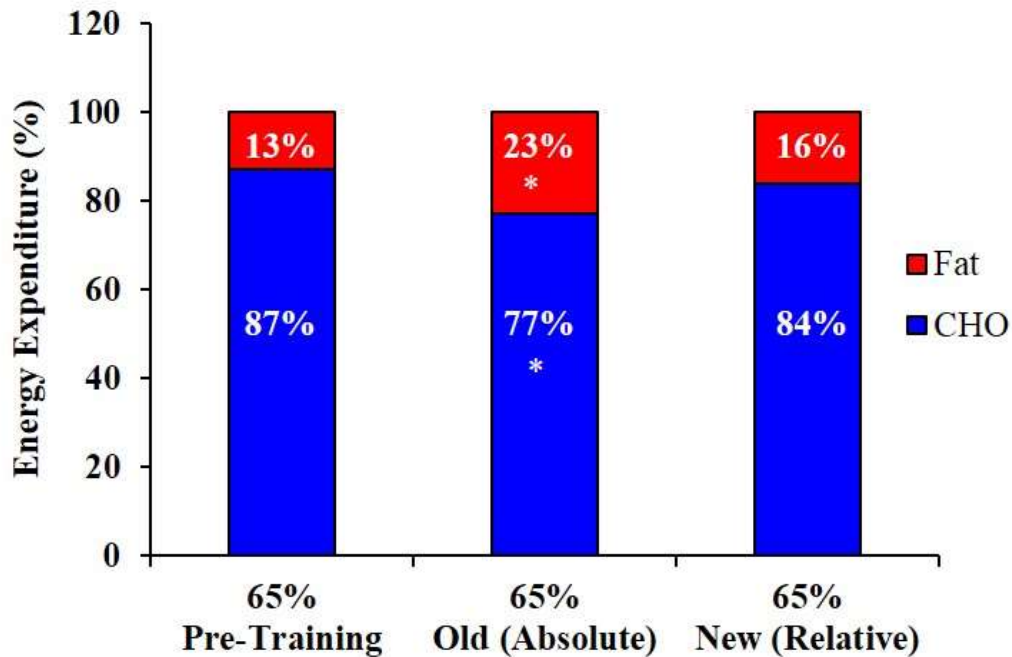
Figure 2.1. Energy expenditure during 60 min of cycling at 65% maximal oxygen uptake, prior to and following nine weeks of cycling training. Data are presented as pre-training intensity, post-training at an absolute workload (old) and post-training at the new relative intensity (new). * Significant change from pre-training. Data from Bergman et al. (1999).

In a second study, Bergman and Brooks (1999) evaluated the interaction of training status and pre-exercise nutritional state on rates of substrate oxidation during graded cycling exercise. In that study, RER values were significantly lower in well-trained compared to untrained individuals during low (22% of $\dot{V}O_2max$) and moderate (40% of $\dot{V}O_2max$) intensity cycling when fasted and also during moderate-intensity exercise when fed or fasted (Bergman & Brooks, 1999). However there was no training effect (i.e., lower RER values), nor any training-nutrient interaction at higher exercise intensities (60 and 75% of $\dot{V}O_2max$). These data demonstrate that because athletes train and compete at exercise intensities > 40% of $\dot{V}O_2max$, they will not oxidise a greater proportion of fat substrates compared with untrained subjects,

regardless of nutritional state. Taken collectively, these results (Bergman & Brooks, 1999; Bergman et al., 1999) demonstrate that the balance of substrate utilisation is unaffected by short periods (~9weeks) of prior endurance training and even during moderate-intensity exercise CHO-derived energy sources are the main fuel source for working muscle. Furthermore, at exercise intensities > 60% of $\dot{V}O_2$max, the relative power output/speed is more important in determining the balance of substrate oxidation than either training or nutritional status. These observations clearly show that when evaluating the effect of prior exercise training on patterns of substrate utilisation, it is critical that the data be placed within the context of the exercise testing paradigm as represented in Figure 2.1.

2.2 Endurance athletes train and race at intensities that are carbohydrate-dependent

A major goal of endurance training for the competitive athlete is to promote physiological and metabolic adaptations that increase the ability to sustain the highest average power output or speed of movement for a given distance (Hawley, 2002; Joyner & Coyle, 2008; Williams et al., 1984). In this regard, endurance training results in an increase in athletes' $\dot{V}O_2$max and also the fractional utilisation of that (higher) aerobic capacity that can be sustained during training and competition (Costill et al., 1973). While the absolute rates of oxidation of all classes of energy substrates increase after training, CHO-based fuels are the predominant energy source for trained muscle when exercise intensities are > 60% of peak oxygen uptake ($\dot{V}O_2$peak). This is because the balance of substrate oxidation at any time during exercise is a function of training-induced adaptations (which promote CHO oxidation) and endurance training-induced adaptations (which promote lipid oxidation), which has previously described by Brooks and Mercier (1994) as the "crossover concept" (Figure 2.2).
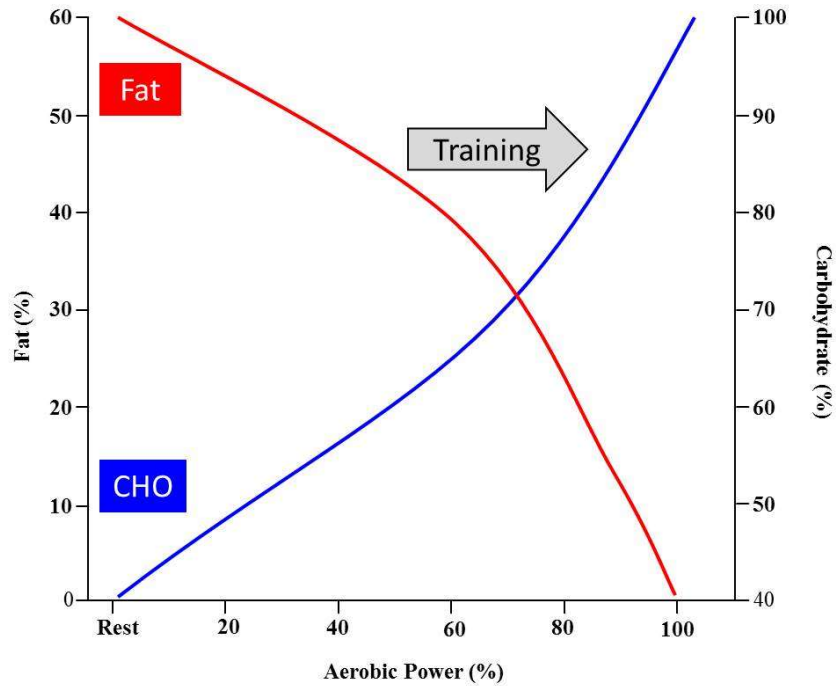
Figure 2.2. The increase in carbohydrate and reduction in fat oxidation associated with an increased relative power output (expressed as a percentage of maximal aerobic power). At the crossover point, a higher relative intensity results in greater carbohydrate dependence. This curve shifts to the right following periods of training at absolute workloads however; the change following training at a relative workload is minimal. Adapted from Brooks and Mercier (1994).

Direct measures of rates of fuel utilisation during field-based training or competition are scarce, particularly in highly-trained athletes. Furthermore, there are only a limited number of laboratory-based investigations that have determined rates of substrate oxidation at the high absolute power outputs/speeds and relative exercise intensities (i.e., > 80% of $\dot{V}O_2$peak) that can be sustained by athletes during training and racing (Hawley et al., 2000; Romijn et al., 1993; Stepto et al., 2002; van Loon et al., 2001). Romijn et al. (1993) investigated the change in energy contribution from endogenous CHO- and fat-based fuel sources during cycling at different exercise intensities. The study findings (Figure 2.3) highlight that the absolute contribution of

energy from circulating substrates (i.e. plasma glucose and FFA) does not change with an increase in exercise intensity. Indeed, the increased energy demand is met from a higher utilisation of intramuscular substrates (i.e. muscle glycogen and IMTG), with muscle glycogen contributing approximately 70% of energy expenditure when cycling at 85% of $\dot{V}O_2peak$.
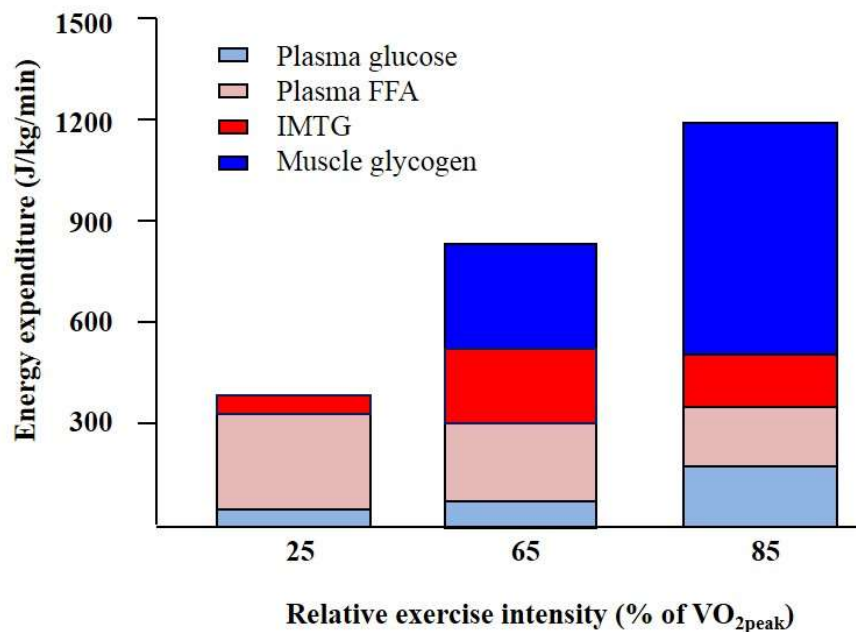
Figure 2.3. Energy expenditure during steady-state cycling at different exercise intensities. The absolute energy contribution from circulating blood glucose and FFA does not change following an increase in the relative exercise intensity. The increased energy demand is met by intramuscular substrates (i.e. IMTG and muscle glycogen). FFA, free fatty acids; IMTG, intramuscular triglycerides. Adapted from Romijn et al. (1993).

With regard to the metabolic demands of endurance cycling training, Stepto et al. (2002) reported that during an interval training session (comprising 8 x 5 min work-bouts at 85% of $\dot{V}O_2$peak, ~325 W), rates of CHO oxidation were ~315 $\mu mol \cdot kg \cdot min^{-1}$ while rates of fat oxidation were ~10-fold lower at ~30 $\mu mol \cdot kg \cdot min^{-1}$. During steady-state cycling at power outputs of 310-320 W (80-85% of $\dot{V}O_2$peak) maintained for ~30 min, rates of CHO oxidation typically range from 300-350 $\mu mol \cdot kg \cdot min^{-1}$, corresponding to RER values of between 0.91 to 0.97 (Hawley et al., 2000; Romijn et al., 1993; van Loon et al., 2001). Recent data from Boorsma et al. (2014) in elite runners ($\dot{V}O_2$peak 80 ± 5 $mL \cdot kg \cdot min^{-1}$, two subjects were Olympians) clearly demonstrate CHO dependence when running at speeds typically undertaken by competitive athletes in training. Boorsma et al. (2014) determined rates of substrate oxidation from RER measures in eight male 1,500 m runners during low- (50% $\dot{V}O_2$peak), moderate- (65% $\dot{V}O_2$peak) and high-intensity (80% $\dot{V}O_2$peak) treadmill running. For the entire group, RER values were 0.85 ± 0.06, 0.89 ± 0.05 and 0.92 ± 0.05 when running at 50, 65 and 80% of $\dot{V}O_2$peak. However, for the top three runners with the highest $\dot{V}O_2$peak values (83.4 $mL \cdot kg \cdot min^{-1}$), RER was greater (0.94) when running at 80% of $\dot{V}O_2$peak than the rest of the group. At this intensity (corresponding to a speed of 19 $km \cdot h^{-1}$, 3:09 $min \cdot km^{-1}$), CHO-based fuels contributed 81% to the total energy cost of running (Boorsma and Spriet, personal communication).

With regard to laboratory-based measures of substrate utilisation during simulated competition, an early investigation reported CHO dependence during simulated marathon running. In that investigation O'Brien et al. (1993) had a group of 'fast' or 'slow' runners complete a treadmill marathon under conditions that would be expected to favour fat oxidation (i.e., non-CHO loaded, overnight fast, no exogenous CHO provision during exercise). The 'fast' runners completed the marathon in 2 h 43 min while the 'slow' runners finished in 3 h 30 min.

Runners in the 'fast' group sustained a significantly greater fractional utilisation of aerobic capacity compared to the slow runners (75 vs. 65% of $\dot{V}O_2$max; $P < 0.05$) resulting in average RER values that were markedly higher (0.99 vs. 0.90; $P < 0.05$) (O'Brien et al., 1993). However, there was no significant difference between 'fast' and 'slow' runners in the total amount of CHO-based fuels oxidised: the higher rate of CHO oxidation in the 'fast' group was compensated by a longer running time in the 'slow' group such that the total CHO combusted was similar (757 vs. 688 g for 'fast' and 'slow' runners respectively). The results of O'Brien et al. (1993) and others (Bosch et al., 1990; Costill, 1970) show CHO dependence during endurance running lasting ~3-4 h.

Given that the current world record for the men's marathon is 2 h 2 min 57 s (an average speed of 20.59 km·h^{-1}, 2:55 min·km^{-1}) it has been proposed that CHO oxidation may be the exclusive source of energy for the working muscles when racing at such velocities (Spriet, 2007). This premise, along with the notion that competitive athletes train at intensities that are CHO-dependent is underpinned by limited laboratory-based measures of substrate utilisation collected from elite runners. Coetzer et al. (1993) compared a range of physiological and metabolic measurements in the fastest nine white and 11 black South African middle- and long-distance runners at the time of investigation. These workers reported that while both groups had similar training volumes, black athletes completed more running at intensities > 80% of $\dot{V}O_2$max (36 vs. 14%). At this intensity (equivalent to a speed of 17 km·h^{-1}) RER values measured during treadmill running were 0.94, indicating 81% of energy was derived from CHO-based fuels. The fractional utilisation of $\dot{V}O_2$max that could be sustained by black athletes was greater than that of white athletes such that at half-marathon pace (21 km·h^{-1}), black athletes could sustain 90% compared to 82% of $\dot{V}O_2$max for the white runners (Coetzer et al., 1993).

When running at 21 km·h^{-1} (2 min 51 sec·km^{-1}) RER values approached 1.0 (total reliance on CHO-based fuels). When exercising at such high intensities, the energy yield per given volume of oxygen is 5.2% higher from CHO- than fat-based fuels (5.058 versus 4.795 kcal respectively) (Krogh & Lindhard, 1920). Indeed, an increase in RER from 0.97 to 1.00 results in a 0.73% increase in energy yield per L of O$_2$ consumed and since the relationship between $\dot{V}O_2$ and speed is linear, this could potentially increase running speed by 0.15 km·h^{-1} and improve the current world marathon record by approximately 50 sec.

With regard to the substrate demands of intense endurance cycling a recent study obtained data for eight competitive cyclists during a series of simulated time-trials (TT) lasting 60, 90 and 120 min and ridden at ≥ 80% of $\dot{V}O_2$max (Torrens et al., 2016). These data show CHO dependence for all TT's independent of duration, with mean RER values of 0.97, 0.96 and 0.94 and mean rates of CHO oxidation 360, 317 and 308 µmol·kg·min^{-1} for 60, 90 and 120 min TT's respectively. Moreover, Cole et al. (2014) have reported that gross mechanical efficiency during prolonged (2 h) cycling is improved and the decrease in efficiency over time attenuated, following three days of a high- (70% of energy) compared to both a moderate- (45% of energy) and low- (20% of energy) CHO diet. While further work with elite athletes is needed to determine metabolic demands of training and racing, it is clear that bioenergetics of sustained, high-intensity endurance exercise is CHO- rather than fat-dependent and that CHO is a more efficient fuel for muscular contraction during intense endurance exercise.

2.3 Altering substrate availability markedly alters patterns of substrate utilisation but does not enhance exercise capacity/performance

The concept of altering substrate availability to modify the pattern of fuel utilisation during exercise dates back over a century when Krogh and Lindhard (1920) first reported that subjects

placed on a high-fat, low-CHO diet for several days had lower RER values during submaximal cycling than when they consumed a CHO-rich diet. Since that time many studies have manipulated fat availability before or during exercise and reported increased rates of fat oxidation and a "sparing" of endogenous CHO reserves, although these effects often fail to translate into improved exercise performance (Burke et al., 2000; Burke et al., 2002; Havemann et al., 2006; Stellingwerff et al., 2006). The issue of high-fat diets and athletic performance has been summarised previously (Burke, 2015) and reviewed extensively elsewhere (Burke & Hawley, 2002; Yeo et al., 2011). However, given the renewed interest in promoting high-fat diets for endurance sport (Noakes et al., 2014; Volek et al., 2015), it is necessary to assess the efficacy of such diets for training and racing in endurance events lasting up to 3 h.

The first modern-day investigation to examine the effects of high-fat diets for athletic performance was that of Phinney et al. (1983). These workers studied five well-trained cyclists who first consumed an energy balanced diet for one week (high-CHO; 35-50 kcal·kg·d^{-1}, 1.75 g protein·kg·day^{-1} with the remainder of energy coming from CHO [66%] and fat [33%]) followed by 28 days of an isoenergetic, high-fat, low-CHO diet (KETO; < 20 g·day^{-1} CHO). Although subjects were requested to continue with their normal training throughout the study, neither objective measures of the volume, intensity or frequency of sessions, nor the subjective ratings of perceived exertion (RPE) were reported. Furthermore, no metabolic parameters were collected during this period. At the end of the dietary intervention period, exercise capacity was assessed by the exercise time to volitional fatigue while cycling at 63% of $\dot{V}O_2max$, and was not different between high-CHO and KETO (147 ± 13 vs. 151 ± 25 min; P = 0.9) (Figure 2.4). However, the average RER during the submaximal ride to exhaustion declined from 0.83 to 0.72 after the KETO diet and this increase in fat oxidation coincided with a three-fold drop in

glucose oxidation and a four-fold reduction in muscle glycogen utilisation (Phinney et al., 1983). The preservation of submaximal exercise capability appears impressive until one examines the individual responses to the dietary interventions which are displayed in Figure 2.4: two subjects performed worse after the KETO diet, one performed the same, while of the two subjects who did improve, one rode substantially longer (148 vs. 232 min) so as to markedly skew the mean time. Of note was that four of the five subjects had a decline in $\dot{V}O_2$max after the KETO diet while RER values at the end of the maximal test dropped from 1.0 to < 0.9 in four subjects.
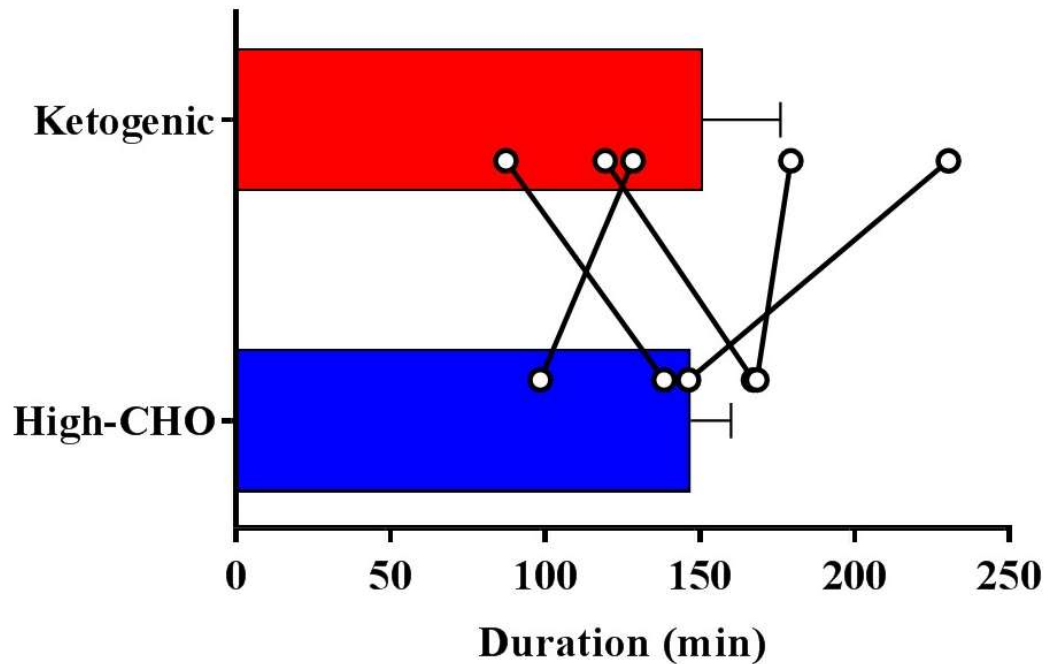
Figure 2.4. Mean and individual cycling time to exhaustion following three weeks of a ketogenic (< 20 g·d^{-1} carbohydrate) or an isocaloric carbohydrate diet (66% energy intake carbohydrate). Data from Phinney et al. (1983).

Recently, Burke et al. (2017) investigated the effects of a ketogenic, low-CHO diet during a three week intensive training block in world class race-walkers. These workers studied 29 athletes who consumed one of three isoenergetic diets: high-CHO diet (HCHO: 8.6 $g \cdot d^{-1}$ CHO, 2.1 $g \cdot d^{-1}$ protein and 1.2 $g \cdot d^{-1}$ fat); periodised diet (PCHO: timing of macronutrient content was altered around training to ensure specific sessions were performed in a low-CHO state); or a low-CHO, high-fat diet (LCHF: < 50 $g \cdot d^{-1}$ CHO, 4.7 $g \cdot d^{-1}$ fat and 2.2 $g \cdot d^{-1}$ protein). Prior to and following the training and dietary intervention, participants completed a graded exercise test consisting of four submaximal stages, each of four min duration. The speed increased by 1 $km \cdot h^{-1}$ and ranged from 11-12 $km \cdot h^{-1}$ to 14-15 $km \cdot h^{-1}$ depending on athlete ability. RER during the graded exercise test declined in the LCHF group from 0.86 to 0.73 and this decrease was observed across all four stages of the test. In addition, participants completed a 25 km standardised walk, which incorporated both laboratory and field testing to enable assessment of substrate utilisation. During this test, whole-body rates of fat oxidation were ~1.6 $g \cdot min^{-1}$ during 2 h of walking at 80% $\dot{V}O_2$peak (i.e. 20 km race intensity) and this was associated with an increased relative O_2 cost during exercise. These are the highest rates of fat oxidation ever to be reported in the literature (Burke et al., 2017). To assess performance, participants completed an International Association of Athletics Federation sanctioned 10 km race and despite a greater ability to utilise fat-based fuel sources and a training-induced increase in $\dot{V}O_2$peak in all dietary groups, 10 km race-walk time only improved in the HCHO and PCHO group (6.6% and 5.3%, respectively) with no improvement in the LCHF group. This lack of performance improvement measured with LCHF, but not HCHO or PCHO was associated in part to the greater O_2 consumption measured during the 25 km standardised walk (i.e. race pace) (Burke et al., 2017).

With regard to the effects of high-fat diets on training capacity, Stepto et al. (2002) reported that subjective RPE was significantly greater after just four days of fat-rich diet compared with an isoenergetic high-CHO diet when well trained cyclists/triathletes undertook standardised laboratory-based interval training. In that study (Stepto et al., 2002), RPE during non-laboratory training was also greater for cycling and all "other training", during the high-fat compared with the high-CHO diet. In an effort to assess the impact of dietary changes on training and daily life, Stepto et al. (2002) administered the Profile of Moods State (POMS) inventory to their subjects at the end of each day of the intervention. The global POMS score was significantly greater after four days of the high-fat compared to the high-CHO diet, while the individual POMS score for fatigue was also higher at this same time point (Stepto et al., 2002). To the best of my knowledge, only one other study has administered the POMS inventory to athletes while consuming a high-fat diet and attempting to maintain their normal training schedule. In that investigation, Keith et al. (1991) reported an elevation in total mood score but no difference for the fatigue component of the inventory when moderately trained female subjects ($\dot{V}O_2$peak 55 mL·kg·min^{-1}) consumed a high-fat, low-CHO diet versus a high-CHO diet for seven days.

As noted previously (Yeo et al., 2011), high-fat 'ketogenic' diet strategies represent as much a low-CHO challenge (i.e., training in the face of low muscle glycogen availability) as a high-fat challenge (i.e., training with high fat availability) and during such interventions muscle (and possibly liver) glycogen content is dramatically reduced. As such, recent interventions have focused on nutritional and training approaches that optimise endogenous CHO stores while concurrently maximising the capacity for fat oxidation during continuous, moderate-intensity exercise (Burke et al., 2000; Burke et al., 2002; Havemann et al., 2006; Stellingwerff et al.,

2006; Yeo et al., 2008). Such "nutritional periodisation" typically encompasses a short-term (i.e., 5-6 days) high-fat diet (60-70% of total energy) in association with an intense training program, followed by 24-36 h of high-CHO intake (70-80% of energy, 'CHO restoration'), as represented in Figure 2.5.

Figure 2.5. Overview of the dietary and training intervention from a series of studies investigating high-fat intake and changes in substrate utilisation and cycling performance. CHO, carbohydrate. (protocol used in (Burke et al., 2000; Burke et al., 2002; Carey et al., 2001; Stellingwerff et al., 2006; Yeo et al., 2008).

Despite the brevity of the fat-adaptation period compared with previous studies (Helge et al., 1998; Lambert et al., 1994; Phinney et al., 1983), ingestion of a high-fat diet results in substantially higher rates of fat oxidation and concomitant muscle glycogen "sparing" during prolonged, submaximal exercise compared with an isoenergetic high-CHO diet consumed for the same time (Burke, 2015; Burke & Hawley, 2002; Yeo et al., 2011). Additionally, as shown in Figure 2.6, higher rates of fat oxidation during exercise persist even under conditions in which CHO availability is increased (i.e., a high-CHO meal before exercise and/or ingesting CHO-containing solutions during exercise) (Burke et al., 2002).
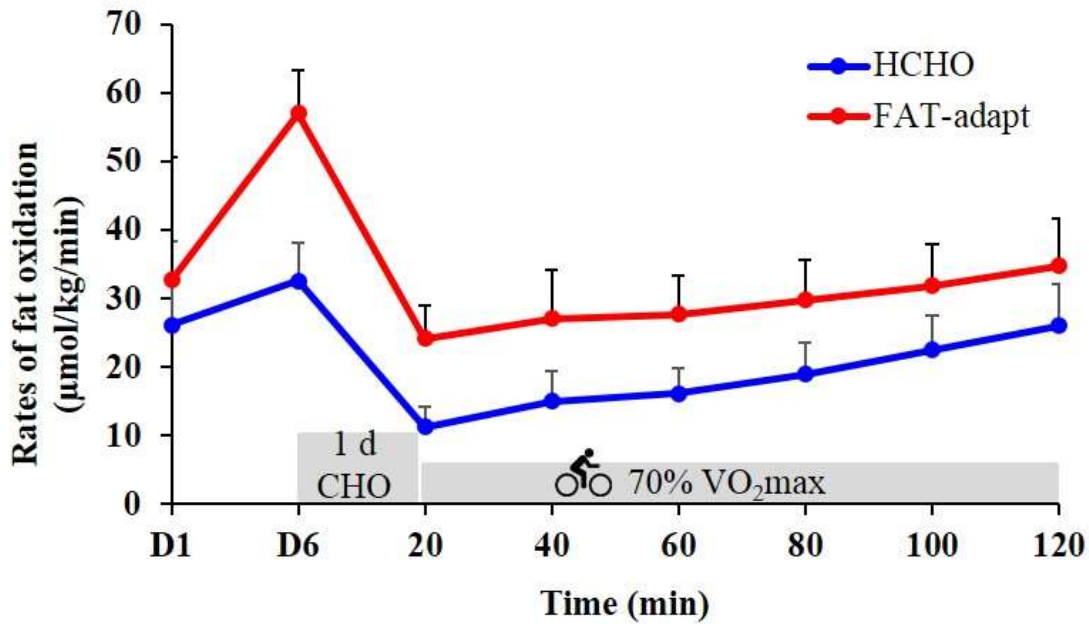
Figure 2.6. Whole body rates of fat oxidation during cycling at 70% maximal oxygen uptake following five days of a high fat (FAT-adapt) or a control high carbohydrate (HCHO) diet with 1 day of carbohydrate restoration. Data are represented as day 1 (baseline), day 6 (after fat adaptation) and during 120 min submaximal steady state cycling on day 7 with exogenous carbohydrate ingestion (after fat adaptation and 1-day CHO restoration). Adapted from Burke et al. (2002).

Yet, despite these robust changes in the patterns of fuel utilisation that favour fat oxidation and "spare" endogenous glycogen utilisation, high-fat, low-CHO diet strategies do not provide any meaningful benefit to the performance of prolonged endurance exercise, nor do they enhance training capacity. This is partly because the high rates of CHO oxidation obligatory to sustain the absolute and relative work-rates typically attained by well-trained athletes during both training and competition when consuming a high-CHO diet are accompanied by increased glycolytic flux that directly inhibits lipolysis and the consequent uptake and oxidation of long-chain FA's (Coyle et al., 1997; Romijn et al., 1995; Spriet, 2014). While fat metabolism is down-regulated in the face of increasing CHO flux/availability and

when moving from moderate to intense exercise, a reciprocal relationship exists to demonstrate that CHO metabolism is down-regulated in the face of increased fat availability. This glycogen "sparing" effect was originally seen as a positive outcome of a high-fat diet but is now recognised as a direct impairment to CHO metabolism and likely underpins some of the reductions in exercise capacity observed after high-fat feeding.

2.4 Effects of altered substrate utilisation on regulatory enzymes and signalling proteins associated with carbohydrate and fat oxidation

Several mechanisms contribute towards the up-regulation of fat metabolism and/or the impaired CHO metabolism following periods of fat adaptation and CHO restoration. These adaptations alter substrate storage, substrate breakdown (i.e. glucose/glycogen or TGs) and rates of whole body substrate oxidation. Burke et al. (2000) have previously reported a reduction in muscle glycogen content following five days of a high-fat diet compared to a high-CHO diet (255 vs 464 mmol$\cdot$kg^{-1} dry wt, respectively). However, following one day of high CHO intake (10 g$\cdot$kg$\cdot$d^{-1}), glycogen content was increased to above baseline in the high-fat condition so that both the high-fat and high-CHO conditions were similar (550-600 mmol$\cdot$kg^{-1} dry wt) (Burke et al., 2000; Yeo et al., 2008). Despite similar resting glycogen content between conditions, muscle glycogen utilisation was ~30% lower in the high-fat compared to high-CHO condition during 2 h of submaximal cycling (70% $\dot{V}O_2$max). This reduction in muscle glycogen breakdown was associated with lower rates of whole-body CHO oxidation in the high-fat compared to high-CHO condition, "sparing" CHO (Burke et al., 2000). In addition to altered CHO storage, high-fat diets elevate IMTG concentration (Helge et al., 2002; Zderic et al., 2004), and this increase persists following CHO restoration (Yeo et al., 2008). The higher whole-body rates of fat oxidation following a high-fat compared to high-CHO diet have been attributed to a

combination of factors including greater IMTG content and higher plasma FFA and glycerol concentration, with the latter indicating greater rates of whole body lipolysis (Zderic et al., 2004).

Various mechanisms underpin these shifts in substrate utilisation, including changes to skeletal muscle proteins which have fundamental roles in substrate transport and breakdown. Firstly, high-fat diets rapidly down-regulate the content of pyruvate dehydrogenase (PDH) in its active form (PDHa) in skeletal muscle at rest (Peters et al., 2001; Peters et al., 1998). This down-regulation is accomplished by rapid up-regulation of the enzyme PDH kinase (PDK) which moves PDH to its inactive form. Collectively these two mechanisms decrease CHO oxidation in the face of sub-optimal CHO availability, a response partly mediated by a reduction in circulating insulin concentration and the increased FFA levels after high-fat feeding (Peters et al., 2001). During submaximal exercise following fat-adaptation (Putman et al., 1993) and one day of CHO restoration (Stellingwerff et al., 2006), PDH activation is reduced both at rest and over a range of exercise intensities. In this regard, the results of Stellingwerff et al. (2006) are important. These workers measured skeletal muscle PDH activity before, during and after 20 min of cycling at 70% $\dot{V}O_2$peak and pre- and post-1 min of maximal sprinting at 150% peak power output (Figure 2.7). Estimations of muscle glycogenolysis from serial biopsies were made during the initial minute of submaximal exercise at 70% $\dot{V}O_2$peak and immediately before and after the 1 min sprint. Despite one day of a high-CHO diet following five days of fat-adaptation, Figure 2.7 shows that resting PDH activity was 50% lower than when subjects consumed a high-CHO diet for six days. During the first minute of submaximal exercise at 70% of $\dot{V}O_2$peak (211 W) rates of muscle glycogenolysis were reduced after fat-adaptation/ CHO restoration compared to the high-CHO condition due to substantially less pyruvate oxidation

(via PDH flux). Even during a maximal one min sprint (~502 W) at a power output that would

be expected to be reliant on CHO-based fuels, rates of glycogenolysis were reduced following

the high-fat diet treatment.
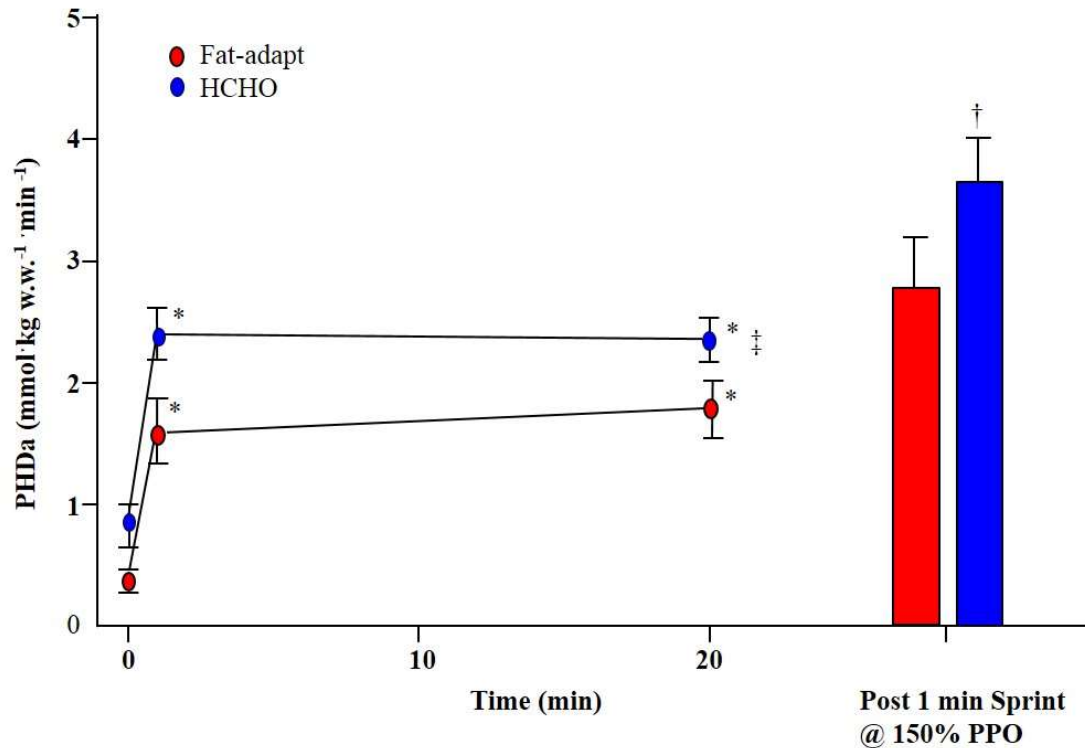
Figure 2.7. Pyruvate dehydrogenase in its active form at rest and during 20 min cycling at 70% peak oxygen uptake followed by a 1-min sprint at 150% peak power output after five days of a high-fat (Fat-adapt) or high-carbohydrate diet (HCHO) and one day of high carbohydrate intake. Values are means ± SE and significance is P < 0.05. * Different from 0 min; ‡ HCHO trial higher than fat-adapt, † different to fat-adapt at time point. Adapted from Stellingwerff et al. (2006).

The suppression of PDH activity and rates of muscle glycogenolysis following high-fat diets impacts directly on high-intensity exercise capacity. Following moderate- and high-intensity aerobic training, maximal PDH activity increases (LeBlanc et al., 2004; Perry et al., 2008) to support the high rates of CHO oxidation that are essential for work rates at > 80% of $\dot{V}O_2$max. Therefore, the persistent down-regulation of PDH activity following fat-adaptation strategies, even in the face of CHO restoration suggests that such dietary interventions are not optimal during exercise in which sustained high-intensity efforts are required. Consistent with the inhibition of resting and exercise-induced PDH activity, Raper et al. (2014) have recently reported slower VO_2 kinetics following six days of a high-fat diet compared with a high-CHO diet.

Direct evidence of a negative effect of high-fat diet on both endurance cycling and race-walking performance comes from Havemann et al. (2006) and Burke et al. (2017), respectively. Havemann et al. (2006) assessed cycling performance after participants had ingested either a high-fat diet (68% energy intake (EI)) or an isoenergetic high-CHO diet (68% EI) for six days followed by one day of CHO restoration. Participants then completed a 100 km laboratory cycle TT (Figure 2.8), designed to simulate aspects of real-life racing, including self-pacing and 1 km and 4 km segments interspersed which required high-intensity sprinting. Although no difference was reported in overall 100 km times (146 min 54 s for high-fat vs. 153 min 10 s for high-CHO) or 4 km sprint performance, power output was lower in the 1 km sprints in the high-fat condition compared to the high-CHO (Figure 2.8). This reduction in power output during 1 km sprints highlights the detrimental effects of a high-fat diet to the strategic activities of a race, such as the breakaways and sprint to the finish line, which are fundamental elements of successful race performance.
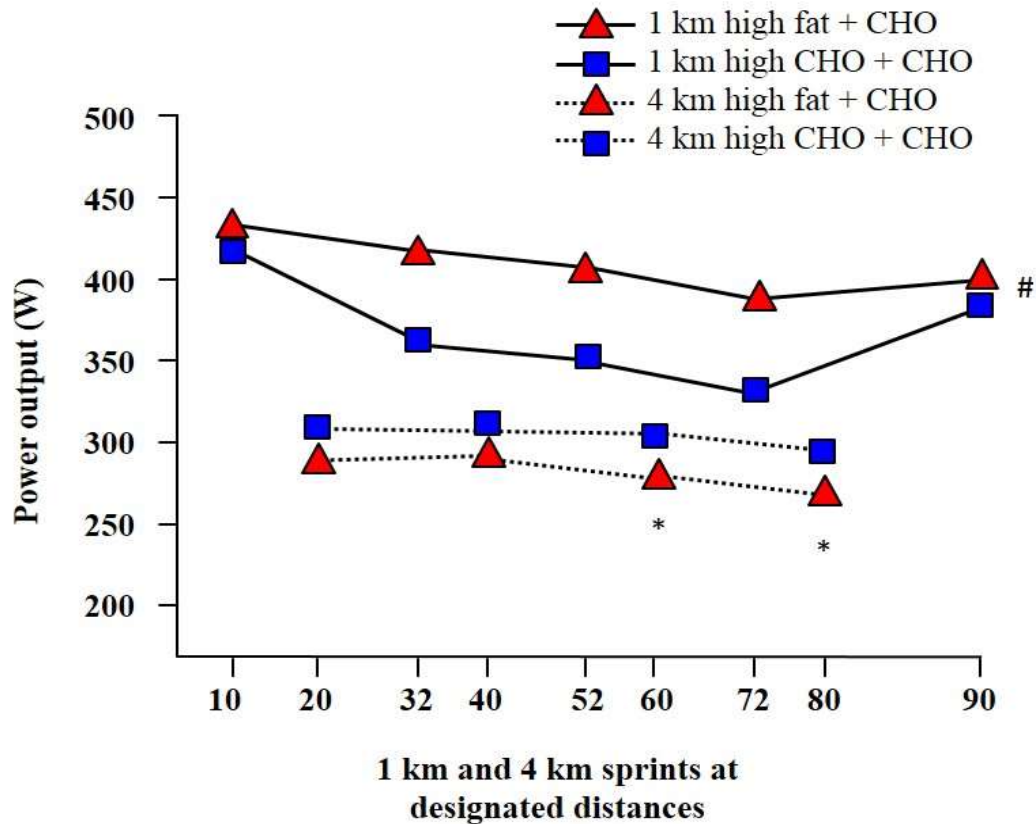
Figure 2.8. Power output during 1 km and 4 km sprints throughout a 100 km cycle, following 6 days of a high-fat or high-carbohydrate diet with 1 day of carbohydrate restoration. # Significant time x condition interaction and significance is $P < 0.05$, * significant effect of time. Adapted from Havemann et al. (2006).

In addition to altering regulatory proteins associated with CHO metabolism, there is evidence that high-fat diets up-regulate FA transporters with roles in transporting FA into skeletal muscle cells and the mitochondria prior to oxidation. Cameron-Smith et al. (2003) measured an increase in fatty acid translocase/CD36 (FAT/CD36) gene expression and protein content following five days of a high-fat diet (> 65% EI from lipids) compared to an isoenergetic high-CHO diet (> 70-75% EI from CHO). FAT/CD36 protein content returned to baseline following one day of CHO restoration, suggesting FAT/CD36 is highly sensitive to substrate availability (Yeo et al., 2008). In contrast to altered FAT/CD36 content, no changes have been

measured in other FA transporters, including gene expression of carnitine palmitoyltransferase-1 (CPT1) or protein abundance of fatty acid binding protein following five days of a high-fat diet (Cameron-Smith et al., 2003). Several other studies have reported increased IMTG concentration following a high-fat diet which indicates that FAT/CD36 has a key role in facilitating FA uptake at the skeletal muscle (Yeo et al., 2008; Zderic et al., 2004).

Yeo et al. (2008) also investigated changes in the energy sensing protein, AMP-activated protein kinase (AMPK) and reported higher resting AMPK activity following fat adaptation with one day of CHO restoration compared to a high-CHO diet alone. However no difference was reported in AMPK protein abundance or the downstream target of AMPK, acetyl CoA Carboxylase (ACC). The mechanisms behind the changes in AMPK activity following a high-fat diet have yet to be elucidated but are likely associated with the slightly reduced glycogen content and a response of well-trained participants completing a period of intensive training on a high-fat diet which is a stark contrast to their typical fuelling strategies (discussed subsequently) (Yeo et al., 2008). Together these alterations to substrate storage, substrate transport and skeletal muscle protein abundance and activity following a high-fat diet provide evidence to support the increased rates of fat oxidation and impaired CHO utilisation during submaximal and high-intensity exercise measured following a high-fat diet (Burke et al., 2000; Burke et al., 2002; Havemann et al., 2006; Stellingwerff et al., 2006).

2.5 Effects of a high fat diet on substrate flux and mitochondrial function

As the 'powerhouse' of the cell, mitochondria play a pivotal role in energy provision via oxidative phosphorylation (OXPHOS) both at rest and during exercise. Given the importance of maintaining ATP concentration within a very narrow range during exercise (25 mM·kg^{-1}; 40-50 g), any modifications to skeletal muscle mitochondria could significantly alter energy

provision during exercise and consequently impact on performance. ATP production in the mitochondria occurs through a series of reactions in the tricarboxylic acid (TCA) cycle, which is located in the mitochondrial matrix, and via the electron transport chain located in the inner membrane of the mitochondria (Krebs, 1970). The electron transport chain comprises five respiratory complexes (complexes I to V) where electrons are transferred along a series of carrier molecules. OXPHOS describes the process where electrons are transported along complexes I to IV of the electron transport chain. These electrons are generated from the reducing agent nicotinamide adenine dinucleotide and flavin adenine dinucleotide, which are produced during the oxidation of nutrients (e.g. glucose or FFAs) (Chance & Williams, 1956). During this process energy is released and a proton gradient is generated across the inner mitochondrial membrane. This electrochemical gradient is used by complex V (CV) to generate ATP (Chance & Williams, 1956; Watt et al., 2010).

Given the importance of the mitochondria and OXPHOS to generate ATP, early studies by Holloszy (1967) investigated the changes to skeletal muscle mitochondria as a consequence of exercise training. Holloszy (1967) was the first to show increased skeletal muscle mitochondrial enzyme activity and electron transport capacity following a period of intense training in rodents, which was associated with greater ATP production. These breakthrough findings led to a host of subsequent studies characterising skeletal muscle mitochondrial adaptations to exercise training. For example, Gollnick and King (1969) identified that increases in mitochondrial enzyme activity following several weeks of exercise training in rodents was associated with an increase in mitochondrial density. It is now well established that exercise training induces mitochondrial biogenesis, indicated by a series of cellular events, including increases in mitochondrial gene and protein expression and synthesis of lipids and proteins following

training (Holloszy & Coyle, 1984; Hood, 2001; Perry & Hawley, 2017). These mitochondrial adaptations and improved ability to generate ATP ultimately underpin 'training adaptation' (Perry et al., 2010).

The work of Perry et al. (2010) provides important insights into the time course of the training induced mitochondrial adaptations following short-term exercise training in humans. These workers had participant's complete a high intensity interval training protocol which included seven intense sessions over a two week period. Figure 2.9 shows that the daily bouts of exercise resulted in transient increases in mRNA, which were subsequently followed by increases in transcriptional and mitochondrial proteins across the intervention (Perry et al., 2010). Increases in protein content of peroxisome proliferator-activated receptor-γ coactivator (PGC1α) occurred in response to the cumulative effects of increases in PGC1α mRNA which preceded increases in maximal citrate synthase activity. These adaptations led to higher "steady-state" levels of protein, which likely contributes towards the improved exercise capacity and altered substrate utilisation following periods of training. Over the two week period, the mRNA response to the training stimulus was reduced (Figure 2.9), which helps explain the 'plateau' in skeletal muscle adaptations often experienced by individuals with a history of training (Hawley & Morton, 2014; Perry et al., 2010).
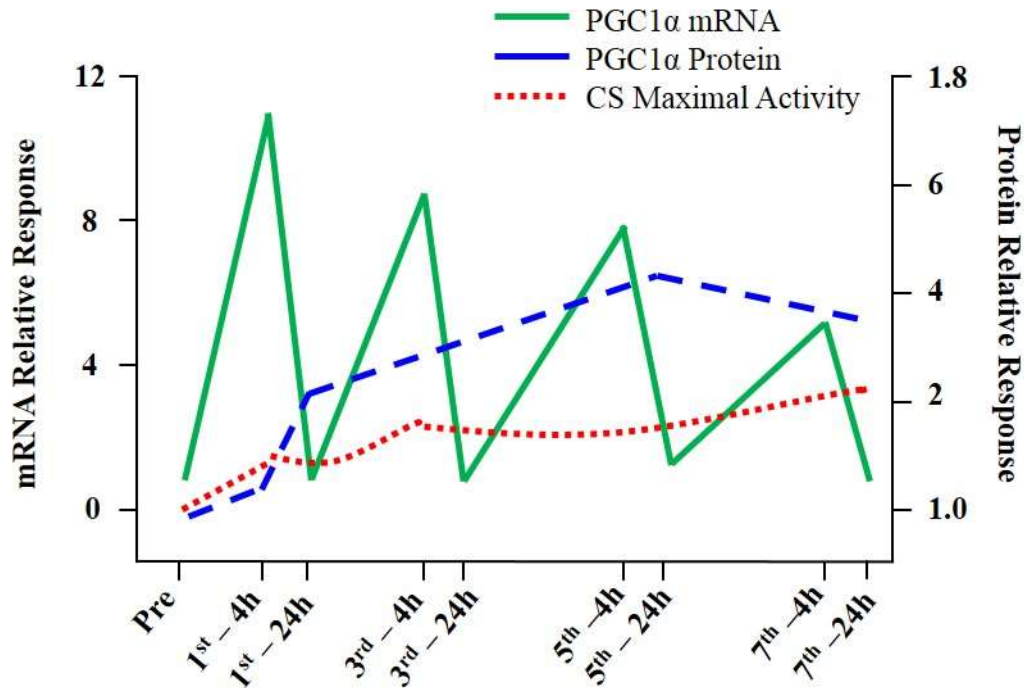
Figure 2.9. Temporal responses and the relationship of peroxisome proliferator-activated receptor-γ coactivator (PGC1α) micro-Ribonucleic acid (mRNA), PGC1α protein and citrate synthase (CS) maximal activity throughout two weeks of high-intensity interval training. Adapted from Perry et al. (2010).

In addition to exercise training, nutritional status prior to and during an exercise bout can significantly alter mitochondrial adaptation because of the highly dynamic nature of mitochondria (Hawley & Morton, 2014; Lionetti et al., 2014). The development of modern analytical techniques, (e.g. Oroborus O2k instrument) has enabled researchers to measure mitochondrial O_2 consumption using high-resolution respirometry in permeabilised muscle fibres. Changes in mitochondrial respiration (i.e. electron transport function) can be measured in response to specific training (Granata et al., 2017) and/or dietary interventions (Skovbro et al., 2011). Changes in mitochondrial respiration are typically investigated relative to the change in mitochondrial content and Larsen et al. (2012) has shown that several biochemical measures including citrate synthase activity and complex I activity have strong associations with

mitochondrial content. In context of this thesis, few studies have examined the changes in mitochondrial respiration in response to exercise-nutrient interventions in humans. Furthermore there are no reports of mitochondrial respiration in well-trained individuals. Skovbro et al. (2011) examined the effect of a high-fat diet (i.e. 16 days; 55-60% fat) versus a 'normal' diet (55-60% CHO) on mitochondrial respiration in permeabilised muscle fibres of untrained individuals. A reduction in mitochondrial electron transport capacity was reported following high-fat feeding however no change in mitochondrial respiration was measured with the 'normal' diet. This decrease in mitochondrial respiration may be associated with previous findings of a reported downregulation in PDHa, a key regulatory enzyme of CHO metabolism and consequently reduced production of acetyl-coenzyme A (Peters et al., 2001; Stellingwerff et al., 2006). PDH has further been identified as a major mechanism regulating metabolic flexibility in isolated skeletal muscle mitochondria in response to altered substrate availability induced by high-fat feeding in rodents (Jorgensen et al., 2017).

In addition to investigating changes in mitochondria following a high-fat diet, Lionetti et al. (2014) have explored the effects of feeding different types of fat (i.e. saturated vs. unsaturated) on mitochondrial function and morphology. These workers examined the dynamic behaviour of mitochondria including changes in mitochondrial fission (i.e. creating new mitochondria and filtering removal of damaged mitochondria) and fusion (i.e. combining damaged mitochondria to maximise function), which are both key processes in maintaining mitochondrial function when cells are faced with metabolic stresses. They showed that high-saturated fat intake was associated with mitochondrial dysfunction and a shift towards mitochondrial fission compared to high unsaturated fat intake which increased mitochondrial fusion (Lionetti et al., 2014) . This research area requires further study to determine if there are further detrimental effects of a

high-fat diet on mitochondrial function and morphology in trained and sedentary populations and how the maladaptation's impact exercise performance and health.

2.6 Overview of ketone metabolism

Although the change in substrate utilisation following a high-fat diet has been well investigated, an area less researched is the concomitant effect of the low-CHO availability over chronic periods of time when ingesting a high-fat diet. The reduction in CHO intake (i.e. < 50 g·day^{-1}) with a high-fat diet stimulates the production of ketone bodies, which may provide an additional fuel source for skeletal muscle and the brain. Ketone bodies have predominately been considered a fuel source to preserve brain and heart function in conditions of prolonged fasting or starvation (Robinson & Williamson, 1980; Veech, 2004). Under such conditions of reduced CHO availability, the increased acetyl-CoA from mobilisation of FAs from adipose tissue results in ketone body production in the mitochondria of the liver (Veech, 2004). The term 'ketone bodies' refers to β-hydroxybutyrate (βHB), acetone and acetoacetate (AcAc), although only AcAc and acetone contain a carboxyl group with two hydrocarbons, making them ketones (Robinson & Williamson, 1980). Although βHB is referred to as a 'ketone', one of its hydrocarbon atoms is replaced by a hydroxyl group. AcAc and βHB are utilised by the brain, heart and skeletal muscle and are therefore commonly measured in the circulation, while acetone is primarily lost via expiration and secreted through urine (Pinckaers et al., 2017). For ketone bodies (i.e. βHB and AcAc) to be utilised as a fuel source, they have to be transported across the plasma and mitochondrial membranes and converted back to acetyl-CoA to be used by the TCA cycle (Robinson & Williamson, 1980) (Figure 2.10).

2.7 Endogenous ketone production

Under conditions of abundant CHO availability, circulating concentrations of ketone bodies are low (i.e. < 0.1 mM), being slightly elevated (0.1-0.5 mM) following an overnight fast (Figure 2.11) (Egan & D'Agostino, 2016; Pinckaers et al., 2017). Prolonged fasting/starvation (~5 days) results in an increase in rates of ketone body production (1-2 mmol·min^{-1} or 140-280 g·day^{-1}), although plasma concentrations typically plateau at ~7-10 mM following five days (Balasse, 1979; Fery & Balasse, 1983). This plateau in circulating ketones is likely an inherent feedback mechanism where ketone bodies limit their own production by stimulating hyperinsulinemia and therefore inhibiting lipolysis (Balasse & Fery, 1989; Bjorntorp & Schersten, 1967; Robinson & Williamson, 1980). Chronic exposure to a ketogenic diet (low-CHO [< 50 g·d^{-1}], low-moderate protein [~15% of EI], high fat [75-80% of EI]) raises plasma ketone concentration to 1-2 mM after several days, with levels reaching the plateau typically achieved by prolonged fasting. Burke et al. (2017) demonstrated a rise in resting ketone concentrations following three weeks of a 'ketogenic' diet (< 50 g·d^{-1} CHO, 78% energy from fat) in highly trained athletes, from < 0.1 mM to ~1.8 mM. However, the ketone concentration reached is highly dependent on the level of CHO restriction and the duration of "keto-adaptation" (Pinckaers et al., 2017).

In addition to altered ketone concentrations as a consequence of altered dietary intake, a rise in circulating ketones is also stimulated by prolonged exercise performed in a fasted state (Egan & D'Agostino, 2016; Pinckaers et al., 2017). For example, Fery and Balasse (1986) reported an increase in circulating ketones to 0.5-1.0 mM during 2 h of exercise performed at 50% V̇O$_2$max following an overnight fast. The extent of the increase in ketone concentration during exercise is dependent upon the training status of the individuals, the relative exercise

intensity, the duration of exercise and the preceding diet (Pinckaers et al., 2017). Evaluating exercise-induced increases in circulating ketones in response to prolonged starvation or overnight fasted is impractical and has little practical relevance to athletes aiming to optimally fuel for training sessions and competition. Furthermore, although high-fat or 'ketogenic' diets increase lipolysis and the release of FFA from the adipose tissue, consequently stimulating an increase in ketone production, such diets also downregulate CHO metabolism and provide little benefit to prolonged exercise performance (Burke et al., 2000; Havemann et al., 2006). As such, ketones bodies derived from exogenous sources may be more practically relevant for athletes to avoid exposure to prolonged fasting and enable the co-ingestion of CHO during training and/or competition. Exogenous ketone sources have recently received attention within the sporting supplement and cycling industry as an alternative fuel source for working skeletal muscle (Abraham, 2015).

2.8 Effects of exogenous ketone ingestion on circulating ketone concentrations

Exogenous forms of ketones have been studied since the 1960's via ingestion of ketone salts administered orally or intravenously (Balasse & Ooms, 1968; Johnson & Walton, 1972). However, ketone salts contain a significant salt load ($\sim$ 1 g·serving^{-1}) and are less effective at increasing circulating ketones when compared to the more recently researched ketone esters (Clarke, 2015; Cox et al., 2016). Ketone esters (i.e. 1, 3-butanediol) do not contain the high salt loads associated with ketone salts and they are also very effective at increasing ketone concentrations in the 1-2 h following ingestion (Clarke et al., 2012; Cox et al., 2016). Ingested ketone esters undergo metabolism in the liver where they are converted to βHB (Figure 2.10) (Cox & Clarke, 2014). Clarke et al. (2012) have recently produced a ketone monoester, R-3-hydroxybutyl R-3-hydroxybutyrate which increases plasma ketone concentrations to ~3 mM

within 1-2 h following ingestion of 400-600 mg·kg^{-1} body mass (BM). However, this response may be altered by concomitant intake of food because of the unique physiological state that this presents (Evans et al., 2017; Pinckaers et al., 2017). As a consequence, the increase in circulating βHB and AcAc concentration following co-ingestion of CHO and a ketone ester is attenuated (~ 1 mM) compared to when ketones are ingested in a fasted state (Stubbs et al. 2015, abstract).
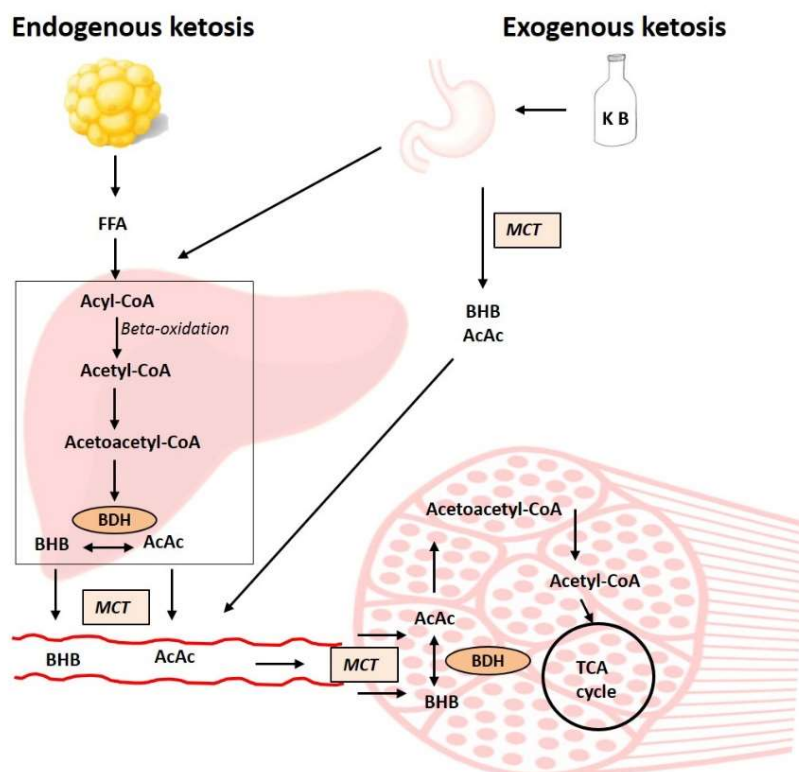
Figure 2.10. Overview of endogenous and exogenous ketone body metabolism in the liver and skeletal muscle. FFA, free fatty acids; BDH, beta-dehydrogenase; MCT, monocarboxylate, AcAc, acetoacetate; βHB, β-hydroxybutyrate; TCA, tricarboxylic acid cycle; KB, ketone bodies.

2.9 Effects of increasing circulating ketones on substrate metabolism

Ketone body metabolism is affected by tissue type (i.e. skeletal muscle vs. brain), the prevailing blood ketone concentrations, sex, training status and also an individual's preceding diet (Pinckaers et al., 2017). The brain utilises ketone bodies in direct proportion to the concentration of this circulating metabolite, which is likely a survival mechanism to preserve brain function in threatening conditions (i.e. starvation) (Evans et al., 2017). In contrast, ketone utilisation in skeletal muscle is a saturable process, which occurs between ~1-2 mM (Evans et al., 2017; Mikkelsen et al., 2015; Pinckaers et al., 2017). The rate of ketone oxidation is highly dependent on whether blood ketone concentrations were attained via endogenous sources or exogenous sources, and appear to be greater in well-trained subjects, which is potentially related to increased activity of enzymes involved in ketone metabolism (Evans et al., 2017; Pinckaers et al., 2017).

Ketone bodies (i.e. βHB and AcAc) have an RQ of 0.89 and 1.00, respectively, similar to that of glucose and thus if appropriate correction factors for CO_2 displacement and urine volume are not used when estimating substrate utilisation, this could lead to an inaccurate representation of data and underestimation of the utilisation of CHO-based fuels (Frayn, 1983; Pinckaers et al., 2017). However, as RQ requires arteriovenous measures which are both complex and highly invasive, this is often not possible with trained participants or non-sterile/lab conditions. Furthermore, increasing ketone body utilisation may impair endogenous CHO availability by reducing liver glucose output and inhibiting PDH and thus pyruvate oxidation, similar to the metabolic changes observed with a high-fat diet (Beylot et al., 1986; Wicklmayr & Dietze, 1978). As high rates of CHO oxidation are necessary for endurance

performance, the downregulation in CHO availability (i.e. muscle and liver glycogen stores) and high circulating ketone bodies may impair exercise capacity.

2.10 Exogenous ketone supplementation: Effects on metabolism and exercise capacity

Despite the potentially detrimental effects of high circulating ketones to CHO metabolism, exogenous ketone ester ingestion has been investigated to determine whether it is the next "magic bullet" for enhancing athletic performance. Clarke et al. (2012) initially investigated the safety, tolerability and pharmacokinetics of an exogenous ketone ester in humans. Participants ingested doses of 420 to 2142 mg·kg^{-1} BM per day for five days. Severe side effects were reported in association with the ketone drink, including vomiting, nausea, flatulence and abdominal pain and two participants withdrew from the study as a result of severe adverse events (Clarke et al., 2012). Clarke and colleagues (2012) utilised this information to complete a series of studies on the effects of the same ketone ester on exercise performance.

Clarke (2015) studied the effect of ketone ingestion (~230 kcal ketone bodies) in trained (elite and sub elite, heavy and lightweight, male and females) rowers prior to a 30 min TT in a fasted state. They showed a 1-2 % improvement in rowing performance following the ketone drink compared to a calorie matched placebo drink (Clarke, 2015). However, athletes do not compete in a fasted state so the relevance of these findings to the athletic population is hard to determine. Cox et al. (2016) subsequently evaluated the effects of ketone ester ingestion on exercise performance in combination with CHO feeding. These workers investigated the effects of either CHO or CHO plus ketone ester ingestion (R-3-hydroxybutyl R-3-hydroxybutyrate, 573 mg·kg^{-1} BM) on blood D-βHB concentrations, substrate metabolism and cycling performance in trained male and female cyclists/triathletes. Participants were overnight fasted

and ingested dose one of the ketone drink 30 min prior to a 60 min steady state (SS) bout of intense cycling (75% maximal workload, 303 W for males, 212 W for females). On completion of the SS cycling, participants ingested dose two of the ketone ester prior to a 30 min TT. Blood D-βHB concentration was significantly higher (ranging between ~1.5- 3 mM) and plasma FFA and blood lactate concentrations significantly lower during SS cycling with the ketone ester plus CHO compared to CHO alone. According to Evans et al. (2017), the optimal range for 'nutritional ketosis' to improve performance when promoted by exogenously ingested ketones is between 1-3 mM (Figure 2.11), therefore the subjects in Cox et al. (2016) successfully reached this range. Following the TT, Cox et al. (2016) found that blood lactate concentration was ~4 mM lower with ketone and CHO ingestion (~14 mM vs. 10 mM) and distance covered during the TT was greater (411 ± 458 m), resulting in a ~2 % performance improvement compared to the CHO only trial. However, there was large individual variation in the performance outcome during the TT following ketone ingestion indicating that some individuals had a positive, and other negative reactions to the supplement.

Therefore, despite evidence that ketone ester ingestion can potentially improve performance, further work is required with study designs that replicate race-day conditions (i.e. following optimal CHO ingestion). Additionally, factors such as tolerability, timing and optimal dose should be considered prior to recommending ketone esters to athletes.
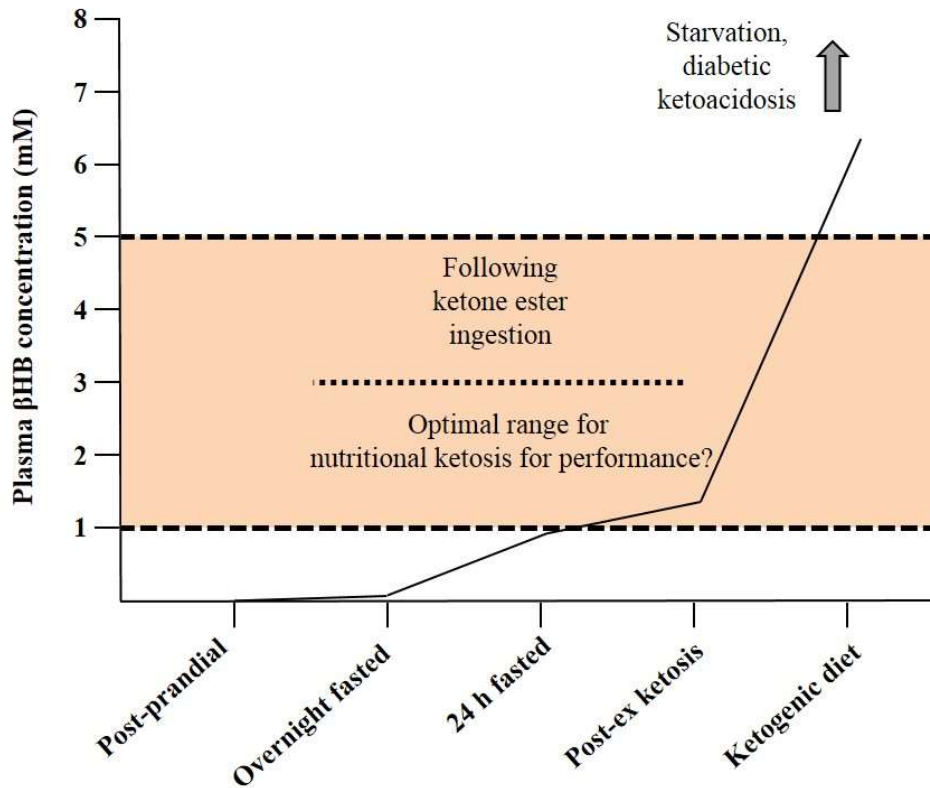
Figure 2.11. Ketone concentration under a range of physiological states. βHB, beta-hydroxybutyrate. Adapted from Evans et al. (2017).

2.11 Summary and directions for further research

The main purpose of training for the enhancement of performance of prolonged (up to 3 h), continuous, high-intensity endurance sport is to promote a range of physiological and metabolic adaptations that permit an athlete to work at both higher absolute and relative power outputs/speeds and delay the onset of fatigue. To meet these goals, competitive endurance athletes perform a large proportion of their daily training at intensities that are close to race-pace and highly dependent on CHO-based fuels for muscle metabolism.

Despite renewed popular interest in high-fat, low-CHO diets for endurance sports; fat-rich diets do not "spare" CHO (i.e., muscle glycogen) or improve training capacity/performance but, instead, induce skeletal muscle adaptations that directly impair rates of muscle glycogenolysis and energy flux. This down-regulation of CHO metabolism underpins the reductions in high-intensity exercise capacity observed after high-fat feeding. However, further work is required to determine the mechanisms underpinning the metabolic perturbations from a high-fat diet, to elucidate whether these adaptations are driven by the high fat or the low-CHO intake.

The limited research on ketone ester ingestion has shown that consuming ketones can increase circulating βHB and AcAc, which act to reduce reliance on CHO based fuels during prolonged, submaximal exercise. Ketone ester ingestion also lowers lactate production during high-intensity cycling and can improve cycling TT and rowing performance when co-ingested with CHO. However further work is required to investigate the effects of ketone ester ingestion on substrate metabolism and athletic performance using ecologically valid designs (i.e. simulating race-like conditions) which translate to typical race day scenarios (i.e. following optimal nutrition guidelines). Additionally, research is required to explore the effects of different types of ketone esters on substrate utilisation and performance and the tolerability/palatability and dose response associated with each ester.

The experimental chapters in this thesis address several of the gaps that have been identified in the literature review. The results from these studies provide novel insights into substrate utilisation in the trained population under conditions of altered substrate availability (i.e. reduced fat availability, high-fat diets, ketone supplementation).

3. Chapter 3- Methodology and design

In keeping with Australian Catholic University guidelines, the methods utilised within each study of this thesis are described in full. In chapters four, five and six the methods section for each study is written as per the guidelines of the respective journal.

3.1 Altering fatty acid availability does not impair prolonged, continuous running to fatigue: Evidence for carbohydrate dependence.

3.1.1   Participants

Twelve competitive male runners who had completed a half-marathon within the previous six months were recruited for this study. Participant characteristics were: age 31 ± 5 (Standard Deviation, SD) y; BM 70.8 ± 5.5 kg; $\dot{V}O_2$max 64.1 ± 3.4 mL·kg^{-1}·min^{-1}; personal best half-marathon time $80{:}50 \pm 4{:}12$ min: s. At the time of the investigation, participants were running $\sim 82 \pm 32$ km·wk^{-1}. Participants were fully informed of all experimental procedures and possible risks before providing their written, informed consent. All participants completed a medical history questionnaire to ensure they were free from illness and injury prior to commencing the performance trials. The study was approved by the Human Research Ethics Committee of the Australian Catholic University.

3.1.2   Preliminary testing and familiarisation

Each participant completed an incremental test to volitional fatigue on a motorised treadmill (Pulsar 3p, HP Cosmos, Nussdorf-Traunstein, Germany) to determine $\dot{V}O_2$max. The test commenced at a speed of 12 km·h^{-1} with a 1% incline and increased by 2 km·h^{-1} every two min until a speed of 16 km·h^{-1} was reached. Thereafter, the treadmill gradient was increased by 2% every two min until the participant reached volitional fatigue, determined as the inability to

maintain the prescribed speed. During the maximal test and the subsequent described performance trials, expired gas was collected via open-circuit spirometry (TrueOne 2400, Parvo Medics, Utah, USA) and the instantaneous rates of O_2 consumption ($\dot{V}O_2$), CO_2 production ($\dot{V}CO_2$) and the RER were calculated every 30 s from conventional equations (Peronnet & Massicotte, 1991). Before each test, gas analysers were calibrated with commercially available gas mixtures (16% O_2, 4% CO_2) and volume flow was calibrated using a 3 L syringe. An individual's $\dot{V}O_2$max was determined as the highest 30 s average which typically coincided with an inability to maintain the prescribed pace, an RER > 1.15 or a subjective rating of maximal effort (RPE). To familiarise participants to the trial protocol they completed a 10 km treadmill run within the 10 days prior to the first performance trial. The treadmill was set at a speed of 95% of individual best half-marathon (21.1 km) time attained in the last 6 months, with a gradient of 1%, to better simulate the metabolic cost of overground running (Bassett et al., 1985). Expired gas was collected at 15 and 30 min and a CHO gel and placebo (PLC) capsules were administered at 25 min.

3.1.3 Overview of study design

In a single blinded Latin square design, each participant completed four performance trials in a randomised order separated by 10-14 d. Participants were blinded to the order of the trials. Each trial required running to volitional fatigue (i.e. the inability to maintain the prescribed speed) at a speed of 95% of their best half-marathon time attained in the last 6 months, with a gradient of 1% (Bassett et al., 1985). The four performance trials were completed following a pre-exercise meal with different nutritional value: CHO ingestion before (2 g $CHO \cdot kg \cdot BM^{-1}$) and during (44 $g \cdot h^{-1}$) (CFED); CFED plus Nicotinic acid (NA) ingestion

(CFED-NA); overnight fasted, PLC meal before and PLC during (FAST); FAST with NA ingestion (FAST-NA).

3.1.4 Exercise and diet control

Participants were instructed to refrain from any vigorous physical activity in the 48 h prior to a performance trial and to abstain from exercise in the 24 h before a trial. During this time, dietary standardisation was achieved by providing participants with individualised pre-packaged meals and snacks (daily intake of 8 g $CHO \cdot kg \cdot BM^{-1}$, 2 g $protein \cdot kg \cdot BM^{-1}$ and 1 g $fat \cdot kg \cdot BM^{-1}$) and by instructing them to abstain from caffeine (i.e. coffee, tea, energy drinks) and alcohol. On the day of a trial, participants were provided a standardised meal consisting of jelly and 600 mL of fluid (2 g $CHO \cdot kg \cdot BM^{-1}$) or a visually identical, taste matched PLC of negligible energy value.

3.1.5 Protocol

On the morning of a performance trial, participants reported to the lab at 0700 h after a 10-12 h overnight fast. A cannula (22G, Terumo, Tokyo, Japan) was inserted into the antecubital vein of the left arm and a baseline blood sample (6 mL) was taken. Following each blood-draw, the cannula was flushed with saline (5 mL NaCl) to keep the vein patent. Participants then ingested either the CHO or PLC meal and rested for 120 min. Further blood samples were taken at -100 min, -12 min and immediately prior (0 min) to the performance trial. NA (10 $mg \cdot kg \cdot BM^{-1}$ or 5 $mg \cdot kg \cdot BM^{-1}$) or PLC (200 mg maltodextrin) capsules were administered 30 min (10 $mg \cdot kg \cdot BM^{-1}$) and 15 min (5 $mg \cdot kg \cdot BM^{-1}$) prior to the performance trial. Participant's BM was recorded prior to completing a 5-10 min warm up on the motorised treadmill at a self-selected pace, which remained the same for each individual for each trial. Participants commenced the performance trial 120 min following breakfast. During the performance trial, participants were

unable to see elapsed time or distance, but were informed to run until they could no longer maintain the prescribed pace.

Blood samples (6 mL), RPE (Borg 1973), heart rate (Polar Electro OY, Kempele, Finland) and expired gas were collected at 20 min intervals. Participants were instructed to inform the principal investigator when they were close to "fatigue", so a final expired gas sample could be collected. Isotonic CHO (SiS GO Isotonic Gel, Blackburn, UK, 44 $g \cdot h^{-1}$ CHO) or PLC gels and NA or PLC capsules were administered every 25 min and 30 min, respectively. Water was consumed *ad libitum* and the total volume consumed throughout each trial measured. On completion of a trial, participants filled out a questionnaire comprising a descriptive 9-point gastrointestinal discomfort scale ("no problem at all" to "worst it's ever been") to rate any distress experienced during the run (Pfeiffer et al., 2010)

3.1.6 Overview of Nicotinic acid ingestion

NA is a pharmacological agent which is primarily used as a cholesterol lowering drug for individuals with hyperlipidemia because of its ability to suppress the release the FFA from triglycerides, ultimately reducing the supply of FFA for hepatic synthesis of very low-density lipoproteins. Previous research has also shown increased NA uptake in the adipose tissue following ingestion and this is explained by a highly expressed NA receptor (Carlson, 2005). In the context of the current study, NA was administered to inhibit the release of FFA from the adipose tissue (i.e. lipolysis) and therefore block the utilisation of this blood-borne fuel source during prolonged, continuous high-intensity running. NA ingestion can cause skin flushing immediately following ingestion and can also be associated with a drop in blood pressure, although the latter is rare (Carlson, 2005). Flushing starts in the face and is at times associated with a warm, itching sensation; however, this is eased approximately 30 min post ingestion.

Intermittent administration of NA was chosen in the current study to minimise the risk of negative circulatory effects which typically occur with a single bolus dose (Pernow & Saltin, 1971). Participants were provided 10 mg·kg·BM^{-1} and 5 mg·kg·BM^{-1} at 30 min and 15 min prior to the treadmill run, respectively, with 5 mg·kg·BM^{-1} administered every 30 min during the half marathon.

3.1.7 Blood analysis

Blood samples (6 mL) were collected into vacutainer's containing EDTA and immediately analysed for blood lactate and glucose concentrations using YSI 2300 STAT Plus^{TM}. Following initial analysis, samples were centrifuged at 1500 g for 10 min at 4 ˚C and aliquots of plasma were stored at -80˚C for later FFA and glycerol analysis. Samples were analysed for FFA concentration using a Non-esterified-fatty acid (NEFA) assay kit (Wako Pure Chemical Industries, Ltd, Osaka, Japan) and glycerol concentration using a glycerol assay kit (Sigma-Aldrich, Ltd, Australia) as per the manufacturer's instructions.

3.1.8 Rates of whole body substrate oxidation and total energy expenditure

Rates of whole body CHO and fat oxidation (g·min^{-1}) were calculated from each steady-state gas sample collected during the performance trial using conventional equations (Peronnet & Massicotte, 1991). The calculations were made from $\dot{V}O_2$ and $\dot{V}CO_2$ measurements using the non-protein RER equations below which are based on the assumption that $\dot{V}O_2$ and $\dot{V}CO_2$ accurately reflect tissue O_2 consumption and CO_2 production.

CHO oxidation (g·min^{-1}) = 4.585 $\dot{V}CO_2$ (L·min^{-1}) - 3.226 $\dot{V}O_2$ (L·min^{-1})

Fat oxidation (g·min^{-1}) = 1.695 $\dot{V}O_2$ (L·min^{-1}) - 1.701 $\dot{V}CO_2$ (L·min^{-1})

Rates of CHO and FA oxidation ($\mu mol \cdot kg \cdot min^{-1}$) were calculated by converting the rates of oxidation ($g \cdot kg \cdot min^{-1}$) to their molar equivalent. It was assumed that six moles of O_2 is consumed and six moles of CO_2 is produced for each mole of CHO (180 g) oxidised and that the molecular mass of human triacylglycerol is 855.3 $g \cdot mol^{-1}$. The molar rates of triacylglycerol oxidation were multiplied by three because each molecule contains three moles fatty acid. Total energy expenditure was estimated for each trial assuming an energy yield of 17.57 kJ and 39.33 kJ for 1 g of CHO and fat respectively.

3.1.9 Statistical analysis

Statistical analysis was undertaken using SPSS (Version 20 for Windows, SPSS Inc, Chicago, IL). Data from the 4 trials were analysed using a linear mixed model (time × treatment). When a significant main effect was reported, a one way ANOVA was used (time or treatment) with Bonferroni post hoc analysis. Statistical significance was set at $P < 0.05$. All data are represented as mean ± SD. Data for distance run was also analysed for magnitude-based effect sizes (ES) between conditions using a custom spreadsheet (Hopkins, 2006). Data was log-transformed to account for non-uniformity and ES ± 90% confidence interval (ES ± 90% CI) calculated and classified as either trivial (-0.2-0.2, ES) small (0.2-0.6 ES), moderate (0.6-1.2 ES) or large (1.2-2 ES). Where the 90% CI overlapped small positive (0.2) and negative (-0.2) values, the effect was considered "unclear".

3.2 Ketone diester ingestion impairs time-trial performance in professional cyclists

3.2.1   Ethical Approval

This study conformed to the standards set by the *Declaration of Helsinki* and was approved (#20161005) by the Ethics Committee of the Australian Institute of Sport (AIS). After comprehensive details of the study protocol were explained to the participants verbally and in writing, all participants provided their written informed consent.

3.2.2   Overview of study design

The study utilised a randomised crossover, double-blind, placebo-controlled design using elite (professional) cyclists attending a pre-season camp at the AIS, Canberra. On two separate occasions, three days apart, participants completed a 20-min standardised warm-up and rested for 5 min prior to completing a 31 km TT on a cycle ergometer (Velotron, Racermate Inc., Seattle, WA, USA). Participants were randomised to consume a 1,3-butanediol acetoacetate diester (described subsequently) (KET) (two doses of 250 mg·kg^{-1} BM) or a viscosity and colour-matched drink (PLAC), given ~30 min before and immediately prior to commencing the warm up. It was not possible to completely replicate the taste of the KET drink, but a comparably novel and bitter-tasting PLAC was prepared from a mixture of flavour essences (rum, almond and bitters Angostura). Pilot testing revealed that the intake of small volume of diet cola immediately after the KET and PLAC was able to quickly mask the taste and texture of the previous drink. In any case, none of the participants had previously ingested a ketone ester supplement and were therefore unable to recognise its characteristics. In recognition of the World Anti-Doping Code under which these cyclists compete, it was

ascertained that ketone supplements are not considered a prohibited substance by the World Anti-Doping Agency.

3.2.3   Participants

Eleven internationally competitive male cyclists (age, 25 ± 7 (SD) y; BM, 73.7 ± 7.6 kg; $\dot{V}O_2$peak, 71.4 ± 5.6 mL·kg·min^{-1}, 5.3 ± 0.3 L·min^{-1}; Maximal Aerobic Power (MAP), 494 ± 20 W) from the ORICA-BikeExchange UCI World Tour (Road Cycling) team participated in this study. Participants included world class elite ($n = 8$) (e.g. 2016 Paris-Roubaix winner, stage medallists from Tour de France, Tour Down Under, Giro d'Italia, Vuelta a España and Australian National championship Time Trial medallists) and highly trained under 23 riders contracted to the team (n=3).

3.2.4   Preliminary Testing and familiarisation

Before commencing the experimental phase, participants visited the laboratory to complete an incremental exercise test and a familiarisation with the cycling ergometer (Velotron, Racermate Inc., Seattle, WA, USA) and the experimental exercise protocol (simulated 2017 World Championships time trial course, Bergen Norway).

3.2.5   Incremental cycling test

Participants completed a 5-min warm-up at 150 W on the cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands), thereafter the test protocol started at 180 W and increased 30 W every 60 s until volitional exhaustion. MAP was determined as the power output of the highest stage completed. If the participant finished partway through a 60 s stage, MAP was calculated in a pro-rata manner. During the maximal test, expired gases were collected into a calibrated and customised Douglas bag gas analysis system, as previously described (Russell

et al., 2002). Peak aerobic capacity ($\dot{V}O_2$peak) was calculated as the highest oxygen consumption recorded over a 30-s period. $\dot{V}O_2$peak and MAP were used to calculate the power output for the individualised warm up on subsequent trial days, described subsequently.

3.2.6   Dietary control

CHO and caffeine intake were standardised the evening before and morning of a trial day and participants were also instructed to abstain from alcohol during the 24 h period prior to a trial day. Participants consumed an evening meal, snack and breakfast prepared by the team chef, providing a CHO content of 2 $g \cdot kg^{-1}$, 1 $g \cdot kg^{-1}$ and 2 $g \cdot kg^{-1}$ BM, respectively. Participants were also provided with a post-exercise recovery drink, 20 min after the completion of the time trial (1 $g \cdot kg^{-1}$ BM CHO and 25 g protein). The composition and timing of all meals was repeated prior to trial two. An example of dietary intake for an 81-81kg athlete is presented in Table 3.1.

Table 3.1. Dietary standardisation including dinner (2 g·kg^{-1} CHO), snack (1 g·kg^{-1} CHO) and breakfast (2 g·kg^{-1} CHO) for an 81-82 kg cyclist.

Food	Quantity
Dinner	
White rice cooked	400 g
100% Orange Juice	600 mL
Honey and soy marinade & sauce	50 mL
Chicken breast grilled	241 g
TimTam Chocolate bites	4 regular biscuits
Snack	
Café raisin toast	2 slices
Strawberry Jam	2 individual serve packets
Breakfast	
White rice cooked	335 g
Eggs whole	120 g
Gatorade	600 mL

3.2.7 Synthesis of ketone ester

The ketone ester synthesised, 1,3-butanediol acetoacetate diester, is a non-ionised sodium-free and pH-neutral precursor of AcAc (D'Agostino, 2012). The ketone ester was synthesised by transesterification of *t*-butylacetoacetate with *R,S*-1,3-butanediol (Savind, Seymour, IL, USA). The resultant product consisted of a mixture of monoesters and diester, the ratio of which could be adjusted by varying the stoichiometry of reactants. After synthesis the crude product was distilled under reduced pressure to remove all solvents and starting materials and the resultant ketone ester was obtained and assessed for purity using gas chromatography-mass spectrometry (GC-MS).

Trial day procedure

3.2.8 Participant preparation

On each of the trial days, participants reported to the laboratory in a rested and overnight (10 h) fasted state, with the timetable creating a ~30 min time between individuals that was repeated on the subsequent trial day. The trial day protocol commenced with the placement of an indwelling cannula (22G; Terumo, Tokyo, Japan) into a cephalic vein to allow for repeat blood sampling while the subject lay in a supine position. A fingertip sample of capillary blood was collected concomitantly with each cannula sample throughout each trial for immediate measurement of blood ketones (ß-hydroxybutyrate; FreeStyleOptium Neo, Abbott Diabetes Care, Doncaster, Australia). Following a resting blood sample (t = 0 min), participants were provided their standardised CHO breakfast including 200 mg caffeine (NO-DOZ Awakeners, Key Pharmaceuticals Pty Ltd, Macquarie Park, Australia), to mimic typical race preparations. Participants were instructed to consume the breakfast within 10 min, with a second blood sample being collected at t = 30 min. At t = 70 min, participants provided a urine sample, were weighed and fitted with a heart rate monitor. At this time, they ingested the first dose (250 mg·kg^{-1} BM) of KET or PLAC, followed immediately by 200 mL diet cola. At t = 90 min participants were seated on the Velotron ergometer, blood samples (4 mL) were collected and participants consumed their second dose in the same manner.

3.2.9 Warm up protocol

Following the second KET or PLAC drink, participants completed a standardised 20-min warm up on the cycle ergometer. The set-up of the bike was performed by team mechanics to replicate each rider's unique bicycle position and was fitted with a calibrated SRM cycling power meter (scientific version, 8 strain gauge, Schoberer Rad Meβtechnik; Jülich, Germany),

set to sample at 1-s intervals (Gardner et al., 2004). The warm up consisted of 3 x 5 min at 50% ventilatory threshold (VT), VT1 and VT1 plus 50% of the difference between VT1 and VT2 (156 ± 14, 312 ± 28, 355 ± 29 W, respectively), followed by 5 min self-paced cycling. Venous and capillary blood samples were collected every 5 min and expired gas was collected continuously during the first 15 min of the warm up. Immediately following the warm up participants, provided a urine sample and ingested an energy gel containing 50 mg caffeine (27 g CHO, PowerBarPowerGel, Nestle, Vevey, Switzerland). During this time (5 min), participants were free to complete their own preparations during which pre-TT blood samples were collected, participants were provided with standard pre-race instructions and the zero offset of the SRM crank was set according to manufacturer's instructions.

3.2.10 Cycling Time-Trial (World Championship Road Cycling Time Trial simulation)

The TT was a simulation of the 2017 Bergen World Championship TT course, based on global positioning system (GPS) mapping data (road altitude and distance) collected by the Orica cycling team staff (M. Quod, unpublished observations). Cyclists completed the 31.17 km TT as fast as possible and during the TT the only feedback provided to the participant was the distance covered (km), cycling gear-ratio (12–27/48-54) and road gradient (%). Participants were only informed of their TT results following the completion of both trials. Heart rate was collected every 5 km and RPE using the Borg 6 to 20 scale and capillary blood samples were collected at 15.74 km and immediately post TT. Participants ingested 250 mL of commercially available 6% CHO drink (Gatorade) at 15.74 km, as this distance corresponded to the point identified by the cyclists as the most appropriate opportunity to drink on the actual course. Samples of venous and capillary blood, and urine, were collected immediately following the TT and participants were weighed. At t = TT + 20 min, blood samples were collected and

participants consumed a recovery drink (2 g·kg^{-1} BM CHO) and continued to rest quietly for a further 40 min. Blood samples were collected at t = TT + 40 min and t = TT + 60 min, with a final urine sample being collected at t= TT + 60 min. Following the removal of the cannula, participants participated in a semi-structured interview with a single researcher using a series of standard questions to probe perceived effort, motivation and comfort rating during the TT. When symptoms (e.g. gut discomfort and problems) were identified, a standardised Likert scale was used to quantify them into mild, moderate or severe levels. On completion of the second trial, participants were asked whether they could identify the trial in which they received the ketone ester, and the trial in which they had performed best. The interview technique was used to probe levels of interest in using a ketone ester supplement in real competition.

3.2.11 Analytical Procedures

Capillary blood samples were analysed for concentration of ketones and lactate (Lactate Pro 2, Akray, Japan). Venous blood samples were collected into 4 mL SST vacutainers with immediate analysis of a small aliquot for blood glucose concentrations (Cobas Integra 400 plus, Roche Diagnostics, Switzerland). This venous sample was then centrifuged at 1,500 g for 10 min at 4 °C, and aliquots of serum were stored at -80 °C for later analysis. Samples were analysed for FFA concentrations using a non-esterified-fatty acid (NEFA) assay kit (Wako Pure Chemical Industries, Ltd, Osaka, Japan), βeta-hydroxybutyrate concentrations using a β-hydroxybutyrate assay kit (Sigma-Aldrich, Ltd, Australia) and AcAc concentrations using an acetoacetate assay kit (Abcam, Cambridge, UK), as per the manufacturer's instructions. Urine samples were analysed for urine ketones (namely AcAc) using ketone reagent strips (Keto-Diastix, Bayer).

3.2.12 Data Analysis

Statistical analysis was completed using SPSS (version 20 for Windows; SPSS, Chicago, IL). Paired t-tests were used to analyse average PO, cadence, heart rate and change in BM in the TT. Blood, urine, PO, hear t rate, cadence, RPE and respiratory data from the two trials were analysed using a linear mixed model (treatment × time) (n = 10) with the exception of respiratory data which includes (n = 9). When analysing respiratory gases, an RER > 1.0 was not included in analysis as participants were not considered to be in steady state (n=1, stage 3 for KET and PLAC). Statistical significance was set at $P < 0.05$ and data is presented as mean ± SD. TT performance was also analysed for magnitude-based effect sizes (ES) between conditions using a custom spreadsheet (Hopkins, 2006). Data were log-transformed to account for non-uniformity and ES with 90% confidence intervals (ES ± 90% CI) were calculated and classified as either trivial (-0.2 to 0.2 ES) small (0.2– 0.6, ES), moderate (0.6 –1.2 ES), or large (1.2–2.0 ES). Where the 90% CI overlapped small positive (0.2) and negative (0.2) values, the effect was considered to be unclear.

3.3 High dietary fat intake increases fat oxidation and impairs skeletal muscle mitochondrial respiration in trained humans

3.3.1   Ethical Approval

This study conformed to the standards set by the *Declaration of Helsinki* and was approved by the Human Research Ethics Committee of Australian Catholic University and registered with the Australian New Zealand Clinical Trials Registry (ACTRN12616000433404). Participants completed a medical history questionnaire to ensure they were free from illness and injury before commencing the study and were informed of all experimental procedures and possible risks prior to providing their written, informed consent.

3.3.2   Overview of study design

Eight well-trained male cyclists with a history of endurance training and riding > 200 km·week^{-1} were recruited for this study. Participant characteristics were: age, 25 ± 4 (SD) y; body mass (BM), 77.3 ± 7.0 kg; $\dot{V}O_2$peak, 64.0 ± 3.5 mL·kg·min^{-1}; peak power output (PPO), 380 ± 36 W. An overview of the study design is shown in Figure 3.1. Each participant completed two experimental conditions in a block randomized, crossover design while undertaking supervised training. There was a ~14 day wash out period between conditions. It was not possible to blind participants to the dietary interventions. However, the principal researchers completing the data collection and performance measures were blinded to the order of experimental trials.

5 days					5 days						1 day	
High-CHO (10 g/kg CHO (73%); 0.8 g/kg Fat (13%))					High-fat diet (4.1 g/kg fat (68%); 2.5 g/kg CHO (18%)) High-PRO (8.8 g/kg protein (67%); 2.6 g/kg CHO (19%))						High-CHO	
Own training	Own training	Own training	Rest	Fed 20 min @ 63 % PPO	Fasted 20 min @ 63 % PPO + HIT	3-4 h long low ride	2-3 h hill ride	Interval training	3-4 h long slow ride		Fasted 20 min @ 63 % PPO + HIT	Fed 100 min @ 63 % PPO + 7 kJ/kg TT

Figure 3.1. Overview of study design including five days high-carbohydrate diet (high-CHO), followed by five days high-fat or high-protein diet (High-PRO) with low-carbohydrate intake and one day of a high-carbohydrate diet. CHO, carbohydrate; PPO, peak power output; HIIT, high intensity interval training; TT, time trial.

3.3.3 Preliminary testing

Each participant completed an incremental test to volitional fatigue on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, Netherlands) to determine $\dot{V}O_2$peak and PPO (Hawley & Noakes, 1992). During the maximal test and all subsequent experimental trials, expired gas was collected every 30 s via open-circuit spirometry (TrueOne 2400; Parvo Medics, Sandy, UT) and the instantaneous rates of O_2 consumption ($\dot{V}O_2$) and CO_2 production ($\dot{V}CO_2$) were used to calculate the respiratory exchange ratio (RER). Before each test, gas analysers were calibrated with commercially available gases (16% O_2, 4% CO_2) and volume flow was calibrated using a 3 L syringe. An individual's $\dot{V}O_2$peak was determined as the highest 30-s average. These data were used to calculate the work rate corresponding to 63% and 80% of PPO for the two experimental rides.

3.3.4 Experimental trials

Participants followed a 'controlled' high-CHO diet (72% EI), 10 g·kg^{-1} BM [HCHO]) for five days prior to an experimental trial (see Table 1). Participants reported to the lab on the 5th day after an overnight fast and a resting blood sample (6 mL) was collected from an antecubital vein. Participants were then provided a standardised breakfast (2 g·kg^{-1} BM CHO). Two hours following breakfast, participants were weighed and a second blood sample was collected before they completed a 20 min continuous ride at 63% PPO. Expired gas and measures of heart rate (HR) and rating of perceived exertion (RPE) were collected during the last 5 min of the ride (Borg, 1973). Water was consumed *ad libitum* and upon completion of the ride, a third blood sample was collected prior to participants leaving the lab for the final (5th) day of the HCHO diet.

The following morning, participants reported to the lab overnight fasted and a cannula (22G; Terumo, Tokyo, Japan) was inserted into an antecubital vein and a resting blood sample (6 mL) was collected. A resting muscle biopsy was then taken from the vastus lateralis using the percutaneous biopsy technique with suction applied (Evans et al., 1982). Participants then repeated the 20 min continuous ride at 63% PPO in the fasted state, before commencing a high-intensity interval session (HIIT) (8 x 5 min at 80% PPO), as previously described (Stepto et al., 2001). The purpose of this interval session was to reduce muscle glycogen stores in both conditions prior to the dietary intervention.

3.3.5   Diet and training intervention

Participants commenced five days of either a high-fat (HFAT) or a high-protein (HPRO) diet (Table 3.3). The HFAT and HPRO diets comprised 67% EI from fat or protein and 19% EI from CHO (Table 6.1). Protein was provided as an alternative macronutrient to meet energy requirements, while CHO was 'clamped'. Total EI was 0.22 MJ$\cdot$kg^{-1} BM. The HFAT diet was comprised of ~55% saturated and 45% unsaturated (mono and polyunsaturated) fats. Fiber intake was matched for both diets. All meals, snacks and energy-containing fluids were provided to participants in pre-prepared packages, with diets individualised for food preference. Participants completed a daily food checklist to maximise compliance and recorded all fluid (water) consumed on a daily basis during both trials. Caffeine ingestion was not permitted 24 h prior to an experimental trial and participants refrained from alcohol during the intervention period. During this time, participants followed a prescribed training program described previously (Burke et al., 2000) that closely matched each individual's habitual road cycle training volume. Training was matched for each experimental treatment and participants were instructed to ride at a rating of perceived exertion (RPE) that corresponded to 11-13 (Borg,

1973) during each on-road session. Participants reported to the lab on day 4 and completed the same HIIT session as on day 1. On the morning of day 6, participants reported to the lab in a fasted state and a resting blood sample (6 mL) and muscle biopsy were collected before they completed a 20 min ride at 63% PPO. Participants were then provided with 1 day of a high-CHO diet (10 g$^.$kg^{-1} BM CHO) (Table 3.2).

3.3.6   *Performance ride*

After an overnight fast participants reported to the laboratory to complete a performance ride consisting of 100 min steady state (100SS) cycling at 63% PPO, followed by a 7 kJ$^.$kg^{-1} BM time trial (TT). On arrival at the laboratory, a cannula was inserted into an antecubital vein and a fasted blood sample (10 mL) was collected. A muscle biopsy was then taken 2-3 cm distal from the previous incision. Participants then consumed breakfast (2 g$^.$kg^{-1} BM CHO) and rested for 120 min. Immediately prior to exercise participants were weighed and a second blood sample was collected. During exercise blood samples (10 mL) and measures of RPE and HR were collected every 20 min, with expired gas collected at 15, 35, 55, 75, and 95 min. Participants were provided with CHO in the form of isotonic gels (SiS GO Isotonic Gel; Blackburn, UK) and a 6% CHO solution (933 mL fluid, 2 gels total) every 20 min throughout the ride at a rate of 60 g$^.$h^{-1} and water was consumed *ad libitum* during each trial. Immediately upon completion of the 100SS ride, a further muscle biopsy was taken. Participants then voided their bladder and had a 3 min rest prior to commencing the TT. Participants were instructed to complete the TT as fast as possible with visual feedback of cadence and verbal feedback of elapsed work as a percentage of the total work (every 10%). Participants were only provided the results of their TT performance upon study completion. Blood samples were collected immediately before and after the TT.

3.3.7 Rates of substrate oxidation and total energy expenditure

Whole body rates of CHO and fat oxidation ($g \cdot min^{-1}$) were calculated from respiratory gas samples collected during rides using the non-protein RER equations (Peronnet & Massicotte, 1991) which are based on the assumption that $V\dot{O}_2$ and $VC\dot{O}_2$ accurately reflect tissue O_2 consumption and CO_2 production:

CHO oxidation ($g \cdot min^{-1}$) = 4.585 $\dot{V}CO_2$ ($L \cdot min^{-1}$) - 3.226 $\dot{V}O_2$ ($L \cdot min^{-1}$)

Fat oxidation ($g \cdot min^{-1}$) = 1.695 $\dot{V}O_2$ ($L \cdot min^{-1}$) - 1.701 $\dot{V}CO_2$ ($L \cdot min^{-1}$).

Rates of CHO and fatty acid oxidation ($\mu mol \cdot kg \cdot min^{-1}$) were calculated by converting the rates of oxidation ($g \cdot kg \cdot min^{-1}$) to their molar equivalent. It was assumed that 6 moles of O_2 is consumed and 6 moles of CO_2 is produced for each mole of CHO (180 g) oxidised and that the molecular mass of human triacylglycerol is 855.3 $g \cdot mol^{-1}$. The molar rates of triacylglycerol oxidation were multiplied by 3 because each molecule contains 3 moles fatty acid.

Table 3.2. Example of high carbohydrate diet (10 g·kg^{-1}, 72% energy intake), based on a 75 kg cyclist.

Food	Quantity
Breakfast	
Sustain cereal	100 g
Milk	400 mL
Just juice orange	200 g
Morning tea	
Sanitarium up n go	500 g
Carmen's muesli bar	90 g
Lunch	
White bread thick sliced	150 g
Coles Sliced ham	50 g
Coles Tasty Light cheese (shredded)	30 g
Afternoon tea	
SiS GO energy bar red berry	40 g
Gatorade	600 mL
Dinner	
Beef Bolognese with pasta	400 g
Lemonade	375 mL
Custard	300 g
Snacks	
Allen's snakes	225 g
Raisin bread	70 g
Jam	2 individual sachets
Gatorade	600 g

Table 3.3. Example of high-protein (8.8 g·kg^{-1}, 67% energy intake (EI)) and high-fat diet (4.1 g·kg^{-1} fat, 70% EI) with low-carbohydrate intake (2.6 g·kg^{-1}, < 20% EI), based on a 75 kg cyclist.

Food High Protein Diet	Quantity	Food High Fat Diet	Quantity
Breakfast		**Breakfast**	
Scrambled eggs (egg whites)	400 g	Scrambled eggs and bacon	200 g
Protein Bread	4 slices	Butter	10 g
High protein milk	400 mL	White bread thick sliced	4 slices
Protein powder (low carb vanilla)	90 g	Jam	2 individual sachets
Lunch		**Lunch**	
Ryvita crackers	40 g	Coconut almond flour bread	1 slice
Tuna, canned sandwich slices	2 tins	Cheese and chive muffin	1 muffin
Protein bread	2 slices	Butter	10 g
Cherry tomato punnet	200 g	Macadamia nuts	30 g
Chobani yoghurt (natural) x2	340 g		
Dinner		**Dinner**	
Turkey breast, rice, vegetables	562 g	Chicken picata	400 g
Protein powder (chocolate)	90 g	Steamed bake vegetables	150 g
		Coconut lemon fat bombs	2 bombs (40 g)
Dessert			
Chobani yoghurt (watermelon and strawberry)	170 g		
Snacks		**Snacks**	
Strawberry whey protein	90 g	Almonds	30 g
Chobani natural yoghurt	170 g	Raisin bread	70 g
High protein milk	600 mL	Chocolate brownie	100 g
Protein gel	4 gels	Butter	10 g
Protein bar (salted caramel)	90 g		

3.3.8 Blood sampling and analyses

Blood samples (6-10 mL) were collected into vacutainers containing EDTA and immediately analysed for blood lactate (YSI 2900 STAT Plus, Yellow Springs, OH, USA) and total cholesterol, high density lipoproteins (HDL), low density lipoproteins (LDL) and triglycerides (Cobas b 101, Roche Diagnostics Ltd, Basel, Switzerland). The remaining sample was then centrifuged at 1,500 g for 10 min at 4 °C, and aliquots of plasma were stored at -80 °C for later analysis of FFA (Wako Pure Chemical Industries, Ltd, Osaka, Japan), glycerol (Sigma-Aldrich, Ltd, Australia), insulin (R-Biopharm – Laboratory Diagnostics Pty Ltd, NSW, Australia), βeta-hydroxybutyrate (βHB) (Sigma-Aldrich, Ltd, Australia) and glucose (Melbourne Pathology, Vic, Australia) concentration.

3.3.9 Mitochondrial respiration analyses

Vastus lateralis muscle biopsies were excised and 10-20 mg was immediately placed into 3 mL of ice-cold biopsy preservation solution (BIOPS) [2.77 mM CaK_2 ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 7.23 mM K_2EGTA, 5.77 mM Na_2ATP, 6.56 mM $MgCl_2 \cdot 6$ H_2O, 20 mM taurine, 15 mM Na_2Phosphocreatine, 20 mM imidazole, 0.5 mM dithiothreitol (DTT), 50 mM MES hydrate; pH 7.1]. Muscle fibers were mechanically separated in ice-cold BIOPS to maximise fiber surface area and transferred into ice-cold BIOPS supplemented with saponin (50 $\mu g \cdot mL^{-1}$) for 30 min with agitation to permeabilise the sarcolemma and allow diffusion of substrates. Fibers were then washed 3 times via agitation in ice-cold MiR05 respiration medium (20 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), 0.5 mM EGTA, 10 mM KH_2PO_4, 3 mM $MgCl_2 \cdot 6H_2O$, 60 mM lactobionic acid, 20 mM taurine, 110 mM D-sucrose, 1 $g \cdot L^{-1}$ bovine serum albumin (BSA); pH 7.1). Fiber bundles were divided and weighed on a microbalance (1.5-3 mg each) for

respirometry analysis in duplicate. All respiration analyses were commenced within 1 h of sampling.

Electron transport system (ETS) and oxidative phosphorylation (OXPHOS) respiration were measured by the Oxygraph O2k high resolution respirometer (Oroboros Instruments, Innsbruck, Austria) via a substrate-uncoupler-inhibitor titration (SUIT) protocol at 37 °C in MIR05 respiration medium with magnetic stirring at 750 rpm. Briefly, after fibers were added and O_2 was injected to the respiration chamber (maintained between 300 and 500 pmol), the sequential addition SUIT protocol commenced with titrations of malate (2 mM final concentration) and octanoylcarnitine (0.2 mM) to determine leak electron-transferring flavoprotein (ETF) respiration. OXPHOS ETF (ETFp) respiration was assessed by addition of adenosine diphosphate (ADP; 5 mM), complex I (CI) substrate pyruvate (5 mM) and complex II (CII) substrate succinate (10 mM). Cytochrome c (10 μM) was added to confirm mitochondrial membrane integrity, and titrations of carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP; 0.025 μM) were added to determine uncoupled respiratory flux. Complex-specific respiration was inhibited by the addition of rotenone (1 μM) and antimycin A (5 μM) to CI and complex III (CIII), respectively. Finally, complex IV (CIV) capacity was measured during oxidation of N,N,N',N'-Tetramethyl-p-phenylenediamine dihydrochloride (TMPD; 0.5 mM) with ascorbate (2 mM). O_2 flux due to auto-oxidation of these chemicals was determined after inhibition of complex IV (CIV) with sodium azide (15 mM) then subtracted from the raw CIV O_2 flux. Chamber O_2 concentration was maintained between 300 and 450 $\mu mol \cdot L^{-1}$. Mass-specific O_2 flux was determined from steady-state flux normalised to tissue wet weight and adjusted for instrumental background and residual O_2 consumption.

3.3.10 Muscle glycogen concentration

Muscle glycogen concentration was determined as described previously (Churchley et al., 2007). In brief, ~20 mg of muscle was freeze-dried and powdered, with all visible connective tissue removed under a microscope. Glycogen was then extracted from the freeze-dried sample and glycogen concentration was determined via enzymatic analysis (Passonneau & Lauderdale, 1974).

3.3.11 Citrate synthase activity

Whole skeletal muscle lysates were prepared at a concentration of 2 mg·mL^{-1} and 5 μL of sample was loaded onto a 96-well microtiter plate with 40 μL of 3 mM acetyl CoA, and 25 μL of 1 mM 5,5'-dithiobis [2-nitrobenzoic acid] (DTNB) in 165 μL of 100 mM Tris buffer (pH 8.3). Subsequently, 15 μL of 10 mM oxaloacetic acid was added to each well and immediately analysed using a SpectraMax Paradigm plate reader (Molecular Devices, Sunnyvale, CA). Absorbance was read at 412 nm and was recorded every 15 s for 3 min after 30 s of linear agitation. Maximal activity was recorded with citrate synthase activity reported in mol·h·kg^{-1} protein.

3.3.12 Protein analyses

For generation of whole skeletal muscle lysates, ~40 mg of skeletal muscle was homogenised in buffer containing 50 mM Tris·HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM DTT, 10 μg/mL trypsin inhibitor, 2 μg·mL^{-1} aprotinin, 1 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride. Samples were spun at 16,000 g for 30 min at 4°C and supernatant was collected. After determination of protein concentration via bicinchoninic acid

protein assay (Pierce, Rockford, IL), lysates were resuspended in Laemmli sample buffer and 10 µg protein of each sample was loaded into 4–20% Mini-PROTEAN TGX Stain-Free Gels (Bio-Rad Laboratories, California, USA). For OXPHOS antibody cocktail, 8.5 µg protein from unboiled lysates were loaded into 12% polyacrylamide gels. Following electrophoresis, gels were activated according to the manufacturer's instructions (Chemidoc; Bio-Rad Laboratories, Gladesville, Australia) and transferred to polyvinylidine fluoride (PVDF) membranes. After transfer, a Stain-Free image was obtained for protein loading normalisation before rinsing membranes briefly in distilled water, blocking for 1 h with 5% nonfat milk, washing three times (5 min each wash) with 10 mM Tris·HCl, 100 mM NaCl, and 0.02% Tween 20 solution (TBST) and incubating with primary antibody diluted in TBST (1:1,000) overnight at 4°C on a shaker. Membranes were incubated for 1 h the next day with a secondary antibody diluted in TBST (1:2,000) and proteins were detected via enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK; Pierce Biotechnology) and quantified by densitometry (Chemidoc; Bio-Rad Laboratories). Time points and both diets for each subject were run on the same gel.

Antibodies against fatty acid translocase (FAT/CD36) (no. 14347), Carnitine palmitoyltransferase-1 (CPT1A) (no. 12252), AMP-activated protein kinase (AMPKα) (no. 2532), phospho-AMPK^{Thr172} (no. 2531), Acetyl CoA Carboxylase (ACC) (no.3662), phospho-ACC^{Ser79} (no.3661), mammalian target of rapamycin (mTOR) (no. 2972), phospho-mTOR^{Ser2448} (no. 2971), S6 Ribosomal Protein RPS6 (no. 2217), phospho-RPS6$^{Ser235/236}$ (no. 2211), Citrate Synthase (no. 14309), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (no. 2118) were purchased from Cell Signaling Technology (Danvers, MA) and total OXPHOS (no. 110411) purchased from Abcam (Cambridge, UK). Volume density of each target band was normalised

to total protein loaded into each lane using Stain-Free technology (Bio-Rad Laboratories), excluding OXPHOS cocktail which was normalised to GAPDH imaged from the same membrane following the addition of stripping buffer (Thermo Fisher Scientific) to the OXPHOS membrane and re-probing for GAPDH. Following protein loading normalisation, each phosphoprotein was then normalised to its respective total protein.

3.3.13 Statistics

Statistical analysis was undertaken using SPSS (Version 20 for Windows, SPSS Inc, Chicago, IL). Data from the two experimental conditions were analysed using a linear mixed model (treatment × time) and subsequent *post hoc* comparisons were completed within the linear mixed model based on least significant difference. Separate analysis was completed to compare day five of high-CHO diet to 100SS (fed) and day one of HFAT or HPRO to five days post-diet (fasted). Normality was visually assessed using the linear model residuals. Differences in TT performance between trials were compared using a Student's paired t-test. Statistical significance was considered at $P < 0.05$. All data are represented as mean ± SD.

4. **Chapter 4- Study 1: Altering fatty acid availability does not impair prolonged, continuous running to fatigue: Evidence for carbohydrate dependence.**

Publication statement:

This chapter is comprised of the following paper published in *Journal of Applied Physiology.*

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4.1 Abstract

This study determined the effect of suppressing lipolysis via administration of nicotinic acid (NA) on fuel substrate selection and half-marathon running capacity. In a single-blinded, Latin square design, 12 competitive runners completed four trials involving treadmill running until volitional fatigue at a pace based on 95% of personal best half-marathon time. Trials were completed in a fed or overnight fasted state: *1*) CHO ingestion before (2 g CHO·kg^{-1}·BM) and during (44 g·h^{-1}) [CFED]; *2*) CFED plus NA ingestion [CFED-NA]; *3*) fasted with placebo ingestion during [FAST]; and *4*) FAST plus NA ingestion [FAST-NA]. There was no difference in running distance (CFED, 21.53 ± 1.07; CFED-NA, 21.29 ± 1.69; FAST, 20.60 ± 2.09; FAST-NA, 20.11 ± 1.71 km) or time to fatigue between the four trials. Concentrations of plasma FFAs and glycerol were suppressed following NA ingestion irrespective of pre-exercise nutritional intake but were higher throughout exercise in FAST compared with all other trials ($P < 0.05$). Rates of whole-body CHO oxidation were unaffected by NA ingestion in the CFED and FAST trials, but were lower in the FAST trial compared with the CFED-NA trial ($P < 0.05$). CHO was the primary substrate for exercise in all conditions, contributing 83-91% to total energy expenditure with only a small contribution from fat-based fuels. Blunting the exercise-induced increase in FFA via NA ingestion did not impair intense running capacity lasting ~85 min, nor did it alter patterns of substrate oxidation in competitive athletes. Although there was a small but obligatory use of fat-based fuels, the oxidation of CHO-based fuels predominates during half-marathon running.

4.2 Introduction

The major goal of endurance training is to induce physiological adaptations that increase an athlete's ability to sustain the highest average power output or speed of movement for a given distance or time (Hawley, 2002), reduce the oxygen cost ($\dot{V}O_2$) of locomotion, and maintain a higher fractional utilisation of maximal oxygen uptake ($\dot{V}O_2max$) during training and competition (Costill et al., 1973). Such adaptations depend, in part, on the rate at which chemical energy (i.e. fat and CHO) can be converted into mechanical energy for skeletal muscle contraction (Hawley, 2002). In most endurance events, a mix of substrates and energy-producing pathways contribute to work outputs and athletes pursue training/dietary strategies that increase the overall capacity of these pathways, as well as implementing acute competition strategies that ensure optimal substrate availability to meet the energy cost of the event.

While the absolute oxidation rate of all energy substrates increases at the high exercise intensities and power outputs sustained by athletes in training and competition, CHO-based fuels are the predominant energy source (Bergman et al., 1999; Brooks & Mercier, 1994; Hawley & Leckey, 2015). However, recent attention has focused on diet-exercise strategies that reduce skeletal muscle dependence on CHO-based fuels (i.e. muscle and liver glycogen, blood glucose, lactate) before and during exercise, while concomitantly maximising fat oxidation (adipose and IMTGs, blood-borne FFAs and TGs) (Volek et al., 2015). It has been proposed that such strategies will enhance performance by promoting greater utilisation of fat-based fuels, whose availability is relatively unlimited (Volek et al., 2015). However, even when these strategies promote rates of fat oxidation that are substantially higher than those achieved by the effects of endurance training alone, there is no clear evidence of a performance benefit (Burke & Kiens, 2006; Havemann et al., 2006; Phinney et al., 1983). Indeed, rates of muscle fat

oxidation are inadequate to support the high relative (70-90% $\dot{V}O_2$max) and absolute work rates sustained by competitive athletes during running or cycling events lasting < 2 h (Hawley & Leckey, 2015; Jeukendrup et al., 2000; Spriet, 2007; Williams et al., 1984).

An alternative strategy to test the role of fat availability to the performance of endurance sports is to investigate scenarios in which the muscle's access to fatty substrates is impaired. As such, several studies have previously measured the effect of suppressing lipolysis via ingestion of the pharmacological agent NA on rates of whole body substrate oxidation However, the majority of these studies have been in cycling and findings may be deemed predictable based on the high-intensity and short duration of the protocols used (Hawley et al., 2000; Murray et al., 1995). Accordingly, in the present study the pharmacological agent nicotinic acid (NA) was administered during simulated half-marathon running in both fed and overnight-fasted states. It was hypothesised that suppressing lipolysis via NA ingestion would not alter substrate selection or have a detrimental effect on half-marathon running capacity since CHO- and not fat-based fuels support optimal endurance exercise up to ~90 min.

4.3 Methods

In keeping with the Australian Catholic University guidelines, the methods for this study are also presented in chapter 3. The methods below are formatted for the *Journal of Applied Physiology*.

4.3.1 Participants

Twelve competitive male runners who had completed a half-marathon race within the previous six months were recruited for this study. Participant characteristics were: age 31 ± 5 (SD) y; BM 70.8 ± 5.5 kg; $\dot{V}O_2$max 64.1 ± 3.4 mL·kg^{-1}·min^{-1}; personal best half-marathon time

80:50 $\pm$ 4:12 min: s. At the time of the investigation, participants were running ~82 $\pm$ 32 km·wk^{-1}. Participants were fully informed of all experimental procedures and possible risks before providing their written, informed consent. All participants completed a medical history questionnaire to ensure they were free from illness and injury prior to commencing the performance trials. The study was approved by the Human Research Ethics Committee of the Australian Catholic University.

4.3.2   Preliminary testing and familiarisation

Each participant completed an incremental test to volitional fatigue on a motorised treadmill (Pulsar 3p, HP Cosmos, Nussdorf-Traunstein, Germany) to determine $\dot{V}O_2max$. The test commenced at a speed of 12 km·h^{-1} with a 1% incline and increased by 2 km·h^{-1} every two min until a speed of 16 km·h^{-1} was reached. Thereafter, the treadmill gradient was increased by 2% every two min until the participant reached volitional fatigue, determined as the inability to maintain the prescribed speed. During the maximal test and the subsequent described performance trials, expired gas was collected via open-circuit spirometry (TrueOne 2400, Parvo Medics, Utah, USA) and $\dot{V}O_2$, $\dot{V}CO_2$ and the RER were calculated every 30 s from conventional equations (Péronnet & Massicotte, 1991). Before each test, gas analysers were calibrated with commercially available gas mixtures (16% O_2, 4% CO_2) and volume flow was calibrated using a 3 L syringe. An individual's $\dot{V}O_2max$ was determined as the highest 30 s average which typically coincided with an inability to maintain the prescribed pace, an RER > 1.15 or a subjective rating of maximal effort (RPE). To familiarise participants to the trial protocol they completed a 10 km treadmill run within the 10 days prior to the first performance trial. The treadmill was set at a speed of 95% of individual best half-marathon (21.1 km) time attained in the last 6 months, with a gradient of 1%, to simulate the metabolic cost of overground running

(Bassett et al. 1985). Expired gas was collected at 15 and 30 min and a CHO gel and placebo (PLC) capsules were administered at 25 min.

4.3.3 Overview of study design

In a single blinded Latin square design, each participant completed four performance trials in a randomised order separated by 10-14 d. Participants were blinded to the order of the trials. Each trial required running to volitional fatigue (i.e. the inability to maintain the prescribed speed) at a speed of 95% of their best half-marathon time attained in the last 6 months, with a gradient of 1% (Bassett et al., 1985). The four performance trials were completed following a pre-exercise meal with different nutritional value: CHO ingestion before (2 g $CHO \cdot kg \cdot BM^{-1}$) and during (44 $g \cdot h^{-1}$) (CFED); CFED plus NA ingestion (CFED-NA); overnight fasted, PLC meal before and PLC during (FAST); FAST with NA ingestion (FAST-NA).

4.3.4 Exercise and diet control

Participants were instructed to refrain from any vigorous physical activity in the 48 h prior to a performance trial and to abstain from exercise in the 24 h before a trial. During this time, dietary standardisation was achieved by providing participants with individualised pre-packaged meals and snacks (daily intake of 8 g $CHO \cdot kg \cdot BM^{-1}$, 2 g $protein \cdot kg \cdot BM^{-1}$ and 1 g $fat \cdot kg \cdot BM^{-1}$) and by instructing them to abstain from caffeine (i.e. coffee, tea, energy drinks) and alcohol. On the day of a trial, participants were provided a standardised meal consisting of jelly and 600 mL of fluid (2 g $CHO \cdot kg \cdot BM^{-1}$) or a visually identical, taste matched PLC of negligible energy value.

4.3.5 Protocol

On the morning of a performance trial, participants reported to the lab at 0700 h after a 10-12 h overnight fast (Figure 4.1). A cannula (22G, Terumo, Tokyo, Japan) was inserted into the antecubital vein of the left arm and a baseline blood sample (6 mL) was taken. Following each blood-draw, the cannula was flushed with saline (5 mL NaCl) to keep the vein patent. Participants then ingested either the CHO or PLC meal and rested for 120 min. Further blood samples were taken at -100 min, -12 min and immediately prior (0 min) to the performance trial. NA (10 mg·kg·BM^{-1} or 5 mg·kg·BM^{-1}) or PLC (200 mg maltodextrin) capsules were administered 30 min (10 mg·kg·BM^{-1}) and 15 min (5 mg·kg·BM^{-1}) prior to the performance trial. Intermittent administration of NA was chosen to minimise the risk of negative circulatory effects which typically occur with a single bolus dose (Pernow & Saltin, 1971). Participant's BM was recorded prior to completing a 5-10 min warm up on the motorised treadmill at a self-selected pace, which remained the same for each individual for each trial. Participants commenced the performance trial 120 min following breakfast. During the performance trial, participants were unable to see elapsed time or distance, but were informed to run until they could no longer maintain the prescribed pace.

Blood samples (6 mL), RPE (Borg 1973), heart rate (Polar Electro OY, Kempele, Finland) and expired gas were collected at 20 min intervals. Participants were instructed to inform the principal investigator when they were close to "fatigue", so a final expired gas sample could be collected. Isotonic CHO (SiS GO Isotonic Gel, Blackburn, UK, 44 g CHO·h^{-1}) or PLC gels and NA or PLC capsules were administered every 25 min and 30 min, respectively. Water was consumed *ad libitum* and the total volume consumed throughout each trial measured. On completion of a trial, participants filled out a questionnaire comprising a

descriptive 9-point gastrointestinal discomfort scale ("no problem at all" to "worst it's ever been") to rate any distress experienced during the run (Pfeiffer et al., 2010)
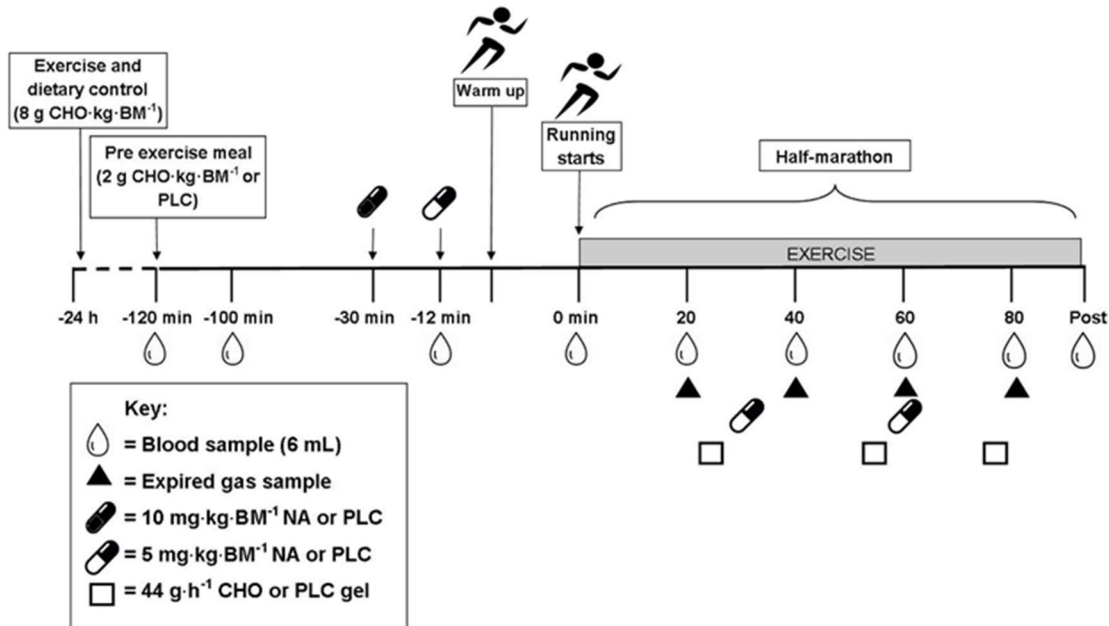
Figure 4.1. Schematic figure of study design. CHO, carbohydrate; PLC, placebo, NA, nicotinic acid.

4.3.6 Blood analysis

Blood samples (6 mL) were collected into vacutainers containing EDTA and immediately analysed for blood lactate and glucose concentrations using YSI 2300 STAT Plus^{TM}. Following initial analysis, samples were centrifuged at 1500 g for 10 min at 4 ˚C and aliquots of plasma were stored at -80˚C for later FFA and glycerol analysis. Samples were analysed for FFA concentration using a Non-esterified-fatty acid (NEFA) assay kit (Wako Pure Chemical Industries, Ltd, Osaka, Japan) and glycerol concentration using a glycerol assay kit (Sigma-Aldrich, Ltd, Australia) as per the manufacturer's instructions.

4.3.7   Rates of whole body substrate oxidation and total energy expenditure

Rates of whole body CHO and fat oxidation (g.min⁻¹) were calculated from each steady-state gas sample collected during the performance trial using conventional equations (Peronnet & Massicotte, 1991). The calculations were made from $\dot{V}O_2$ and $\dot{V}CO_2$ measurements using the non-protein RER equations below which are based on the assumption that $\dot{V}O_2$ and $\dot{V}CO_2$ accurately reflect tissue O_2 consumption and CO_2 production.

CHO oxidation (g·min⁻¹) = 4.585 $\dot{V}CO_2$ (L·min⁻¹) - 3.226 $\dot{V}O_2$ (L·min⁻¹)

Fat oxidation (g·min⁻¹) = 1.695 $\dot{V}O_2$ (L·min⁻¹) - 1.701 $\dot{V}CO_2$ (L·min⁻¹)

Rates of CHO and fatty acid oxidation (μmol·kg·min⁻¹) were calculated by converting the rates of oxidation (g·kg·min⁻¹) to their molar equivalent. It was assumed that six moles of O_2 is consumed and 6 moles of CO_2 is produced for each mole of CHO (180 g) oxidised and that the molecular mass of human triacylglycerol is 855.3 g·mol⁻¹. The molar rates of triacylglycerol oxidation were multiplied by three because each molecule contains three moles fatty acid. Total energy expenditure was estimated for each trial assuming an energy yield of 17.57 kJ and 39.33 kJ for 1 g of CHO and fat respectively.

4.3.8   Statistical analysis

Statistical analysis was undertaken using SPSS (Version 20 for Windows, SPSS Inc, Chicago, IL). Data from the four trials were analysed using a linear mixed model (time × treatment). When a significant main effect was reported, a one way ANOVA was used (time or treatment) with Bonferroni post hoc analysis. Statistical significance was set at $P < 0.05$. All data are represented as mean ± SD. Data for distance run was also analysed for magnitude-based ES between conditions using a custom spreadsheet (Hopkins, 2006). Data was log-transformed

to account for non-uniformity and ES ± 90% confidence interval (ES ± 90% CI) calculated and classified as either trivial (-0.2-0.2, ES) small (0.2-0.6 ES), moderate (0.6-1.2 ES) or large (1.2-2 ES). Where the 90% CI overlapped small positive (0.2) and negative (-0.2) values, the effect was considered "unclear".

4.4 Results

Twelve participants commenced this study but one participant was unable to complete the FAST trial due to illness, while another participant did not complete two of the prescribed performance trials with NA ingestion (CFED-NA and FAST-NA) due to side effects (i.e. dizziness, abdominal cramps). The pre-exercise data for the latter two trials have been included in analyses. Post-hoc power analysis calculations showed this study was 85% powered for the desired outcomes.

4.4.1 Running distance covered

There were small but statistically non-significant differences in the distance run such that CFED > CFED-NA > FAST > FAST-NA (Figure 4.2; P = 0.067). ES statistics revealed a moderate reduction in distance run in FAST-NA (ES -0.96 ± 0.61) compared to CFED and a small reduction in FAST compared to CFED (ES -0.54 ± 0.65). The difference in distance run in CFED vs CFED-NA and FAST vs FAST-NA was "unclear" (ES -0.24 ± 0.64; -0.16 ± 0.53 respectively). No difference was measured for the time to completion between trials (Table 4.1; P = 0.053).
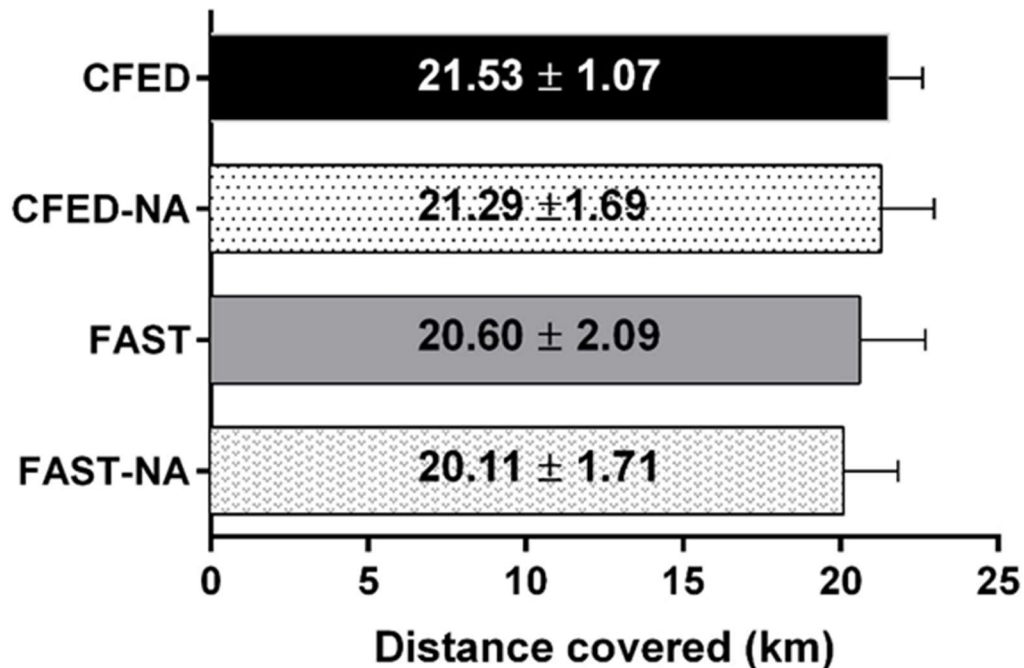
Figure 4.2. Running distance covered (km) during experimental trials. CFED, carbohydrate trial; CFED-NA, carbohydrate with nicotinic acid trial; FAST, fasted trial; FAST-NA, fasted with nicotinic acid trial. Values are means ± SD.

4.4.2 Blood metabolites

A significant treatment × time interaction was observed for both plasma FFA ($P < 0.001$) and plasma glycerol concentrations ($P < 0.01$) from rest until post exercise (Figure 4.3). There was no difference in FFA or glycerol concentrations at rest between treatments. The ingestion of NA suppressed lipolysis and blunted the typical exercise-induced increase in FFA concentrations in the CFED-NA and FAST-NA trials. Following the onset of exercise, FFA concentrations remained higher in the FAST trial compared to the CFED, CFED-NA and FAST-NA trials until the completion of exercise (Figure 4.3A; $P < 0.05$). FFA concentrations increased in the CFED trial between 60 and 80 min of exercise ($P < 0.05$) but such an increase was not

observed in the CFED-NA trial. FFA concentrations were lower in the CFED than the FAST trial post exercise (0.29 ± 0.05 vs. 0.50 ± 0.21 mM respectively, $P < 0.001$). Following 20 min of exercise, glycerol concentrations remained higher in the FAST trial than the CFED, CFED-NA and FAST-NA trials until exercise completion (Figure 4.3B; $P < 0.05$). Increases in glycerol concentrations during the first 40 min of exercise were similar in the CFED, CFED-NA and FAST-NA trials. From 60 min of exercise, glycerol concentrations continued to elevate in the CFED trial until post exercise (0.46 ± 0.16 to 0.54 ± 0.18 mM, $P < 0.05$), such that they remained significantly higher than the CFED-NA trial during this period ($P < 0.01$).

A significant treatment $\times$ time interaction was observed for blood glucose and lactate concentrations (Figure 4.4; $P < 0.001$). Glucose concentrations increased above rest following the ingestion of a CHO meal in the CFED and CFED-NA trials (Figure 4.4A; CFED: 1.80 ± 0.39; CFED-NA: 1.67 ± 0.50 mM, $P < 0.001$). Thereafter a decrease in glucose concentrations to below rest was observed in the CFED and CFED-NA trials until exercise commenced ($P < 0.001$). At 20 min of exercise, glucose concentrations were lower in the CFED and CFED-NA trials compared to the FAST and FAST-NA trials ($P < 0.02$). In all 4 trials, glucose concentrations increased until 40 min of exercise and remained relatively stable thereafter until post exercise (Figure 4.4A).

For all performance trials, lactate concentrations increased in the first 20 min of exercise above baseline (Figure 4.4B), where FAST was lower than CFED, CFED-NA and FAST-NA trials ($P < 0.02$) and CFED-NA was higher than the CFED trial ($P < 0.02$). From 20 to 80 min of exercise no change was observed in lactate concentrations in the CFED, FAST and FAST-NA trials, although there was a decrease in the CFED-NA trial (3.24 ± 0.68 to 2.54 ± 1.24

mM, P < 0.001). No difference was observed in post-exercise lactate concentrations between treatments.
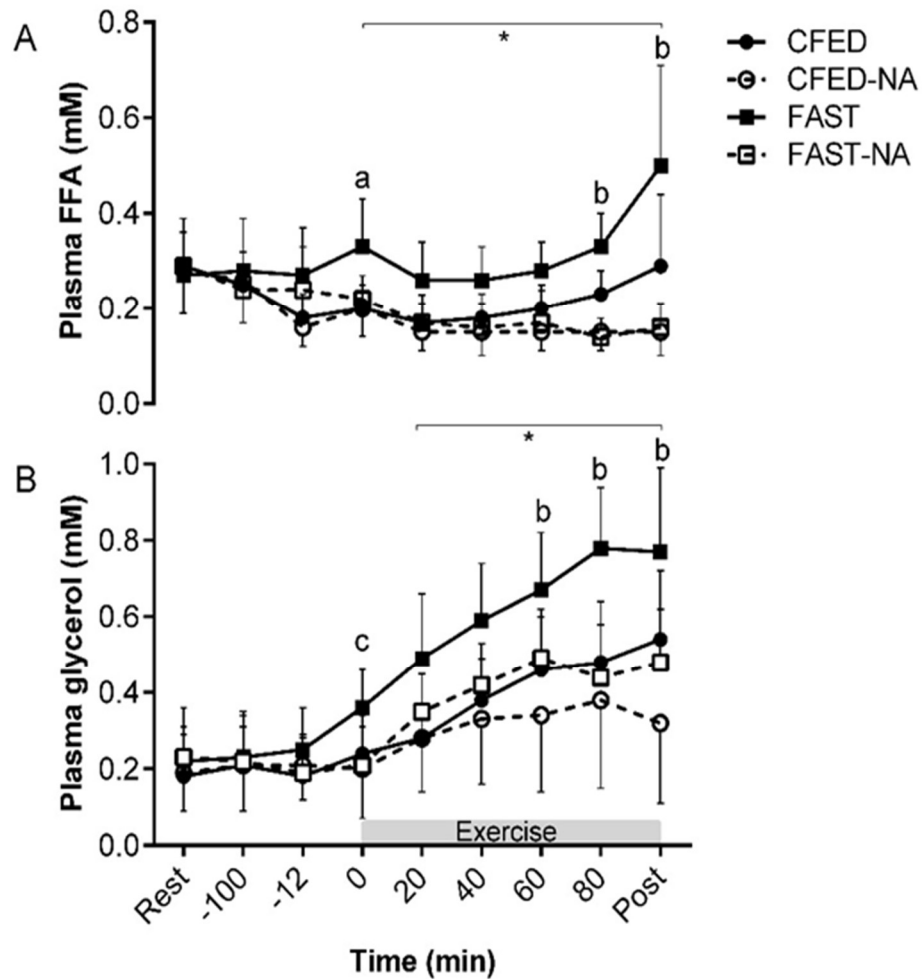
Figure 4.3. Plasma free fatty acid (A) and glycerol concentrations (B) during all experimental trials. CFED, carbohydrate trial; CFED-NA, carbohydrate with nicotinic acid trial; FAST, fasted trial; FAST-NA, fasted with nicotinic acid trial; FFA, free fatty acids. Values are means ± SD. Significantly different (P < 0.05), *FAST to CFED, CFED-NA, FAST-NA; a CFED, CFED-NA, FAST-NA to rest, b CFED to CFED-NA, c FAST to rest.
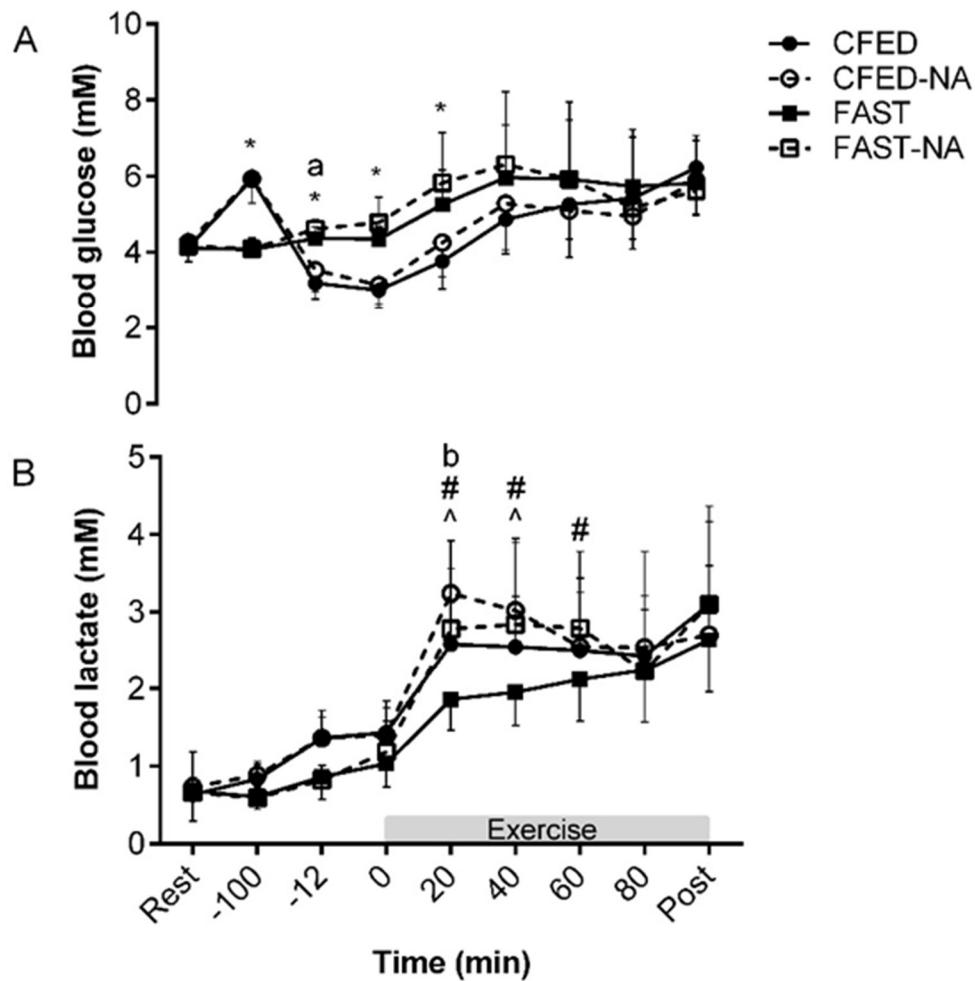
Figure 4.4. Blood glucose (A) and lactate (B) concentrations during all experimental trials. CFED, carbohydrate trial; CFED-NA, carbohydrate with nicotinic acid trial; FAST, fasted trial; FAST-NA, fasted with nicotinic acid trial. Values are means ± SD. Significantly different ($P < 0.05$), *CFED & CFED-NA to FAST & FAST-NA; a CFED, CFED-NA, FAST-NA to rest, b CFED to CFED-NA, # FAST to FAST-NA, ^ FAST to CFED, CFED-NA, FAST-NA.

4.4.3 CHO and fat oxidation during exercise

Rates of whole body CHO oxidation were similar in the CFED, CFED-NA and FAST-NA trials, but were lower in FAST compared to the CFED-NA trial (338.48 ± 34.71 vs. 297.15

± 45.88 umol·kg·min^{-1}, respectively, P = 0.010), such that there was a main treatment effect (P = 0.007) (Table 4.1). Rates of fat oxidation were higher in the FAST trial compared to the CFED-NA trial (16.78 ± 8.74 vs. 8.92 ± 6.65 umol·kg·min^{-1}, P = 0.023) and there was a main effect of treatment (P = 0.008). No difference in fat oxidation was observed in the CFED, CFED-NA and FAST-NA trials.

There was a significant effect of treatment for total CHO oxidised during each trial (P < 0.001) but no difference for total fat oxidised. Total CHO oxidation was lower in the FAST trial in comparison to the CFED and CFED-NA trials (310.22 ± 49.95 vs. 358.48 ± 46.36 vs. 371.89 ± 27.06 g, P = 0.025, P = 0.002 respectively). Estimated total energy expenditure was lower in the FAST trial than the CFED-NA trial (6539 ± 747 vs. 7164 ± 609 kJ, P = 0.011), as such there was a significant effect of treatment (P = 0.010) on estimated total energy expenditure.

4.4.4 Respiratory parameters and RPE

There was a main effect of time (P = 0.042) and treatment (P = 0.004) for RER (Figure 4.5). RER was lower in the FAST trial compared to the CFED-NA trial (0.94 ± 0.03 vs. 0.97 ± 0.02, P = 0.016) although no difference in RER was observed within the CFED (0.96 ± 0.03) and CFED-NA trials or the FAST and FAST-NA (0.96 ± 0.02) trials. There was no difference in relative exercise intensity between the four trials (P = 0.137) (Table 4.2). There was a main effect of time for $\dot{V}O_2$ and $\dot{V}CO_2$, heart rate, RR and RPE for all trials (P < 0.05), but no treatment effect for these variables (Table 4.2). $\dot{V}O_2$ and $\dot{V}CO_2$ increased in the 4 trials from 60 min to exercise completion (3.55 ± 0.38 to 3.62 ±0.38, 3.38 ± 0.36 to 3.46 ± 0.40 L.min^{-1} respectively, P < 0.05) and heart rate, RR and RPE increased from 20 min to exercise completion (165 ± 8 to 173 ± 9 bpm, 44 ± 6 to 51 ± 9 bpm, 13 ± 1 to 17 ± 2, respectively, P < 0.05).

4.4.5 Fluid intake, body mass loss and gastrointestinal distress

There were no differences in the average fluid consumed (330 ± 171 mL, P = 0.680) or

loss in BM (1.73 ± 0.32 kg, P = 0.081) during the 4 experimental trials. No significant difference

was reported between trials (P = 0.241), with gastrointestinal stress rated as 'no problem at all'

in the CFED and FAST trials to 'very very minor' in the CFED-NA and FAST-NA trials.
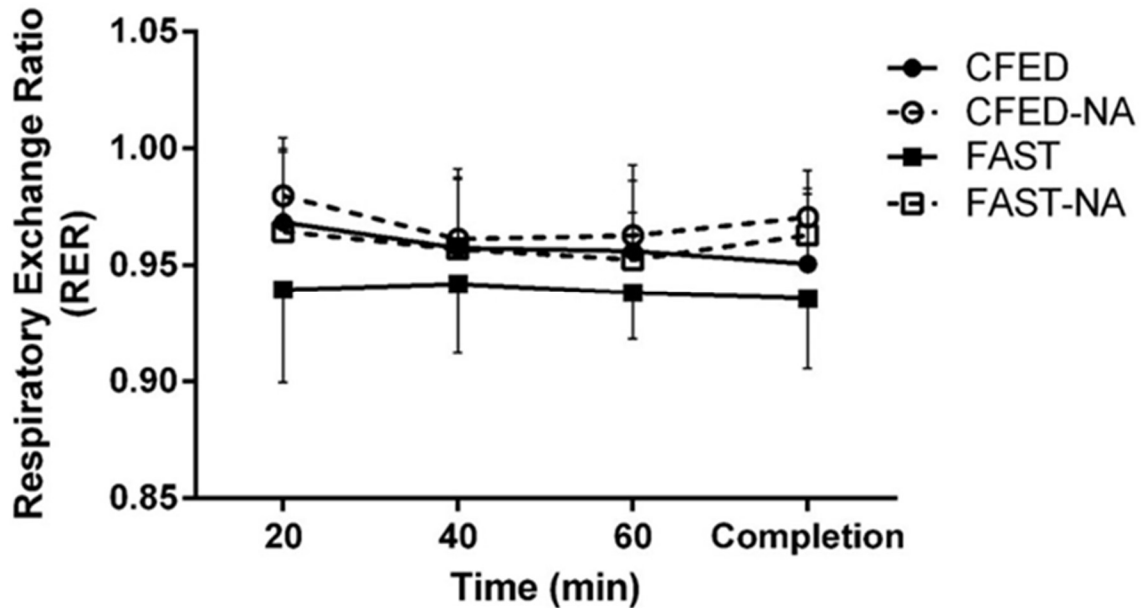
Figure 4.5. Respiratory exchange ratio during all experimental trials. CFED, carbohydrate trial; CFED-NA, carbohydrate with nicotinic acid trial; FAST, fasted trial; FAST-NA, fasted with nicotinic acid trial. Values are means $\pm$ SD. Significantly different between treatments (P < 0.05), *CFED-NA to FAST.

Table 4.1. Metabolic responses for the four experimental trials.

Treatment	CHO (g·min⁻¹)	Fat (g·min⁻¹)	CHO (μmol·kg.min⁻¹)	Fat (μmol·kg.min⁻¹)	Total CHO (g)	Total Fat (g)	Total Energy Expenditure (kJ)
CFED	4.15 ± 0.57	0.25 ± 0.18	322.02 ± 43.77	11.82 ± 8.51	$358.48 \pm 46.36^*$	20.98 ± 13.64	7123 ± 804
CFED-NA	$4.36 \pm 0.46^*$	$0.19 \pm 0.15^*$	$338.48 \pm 34.71^*$	$8.92 \pm 6.65^*$	$371.89 \pm 27.06^*$	16.02 ± 11.26	$7164 \pm 609^*$
FAST	3.80 ± 0.70	0.34 ± 0.17	297.15 ± 45.88	16.78 ± 8.74	310.22 ± 49.95	27.68 ± 14.14	6539 ± 747
FAST-NA	4.17 ± 0.57	0.23 ± 0.16	324.28 ± 38.04	11.34 ± 7.47	337.43 ± 35.71	18.62 ± 12.35	6661 ± 769

CFED, carbohydrate trial; CFED-NA, carbohydrate with nicotinic acid trial; FAST, fasted trial; FAST-NA, fasted with nicotinic acid trial. Values are means $\pm$ SD. *Significantly different to FAST trial ($P < 0.05$).

Table 4.2. Respiratory parameters, RPE and average run time until completion for the four experimental trials.

Treatment	$\dot{V}O_2$ (L·min^{-1})	$\dot{V}CO_2$ (L·min^{-1})	HR (bpm)	RR (bpm)	RPE	Time (min)	% $\dot{V}O_2$max
CFED	3.57 ± 0.42	3.41 ± 0.38	167 ± 9	46 ± 9	14 ± 1	1:26:32 ± 0:06:01	78.5 ± 3.7
CFED-NA	3.61 ± 0.39	3.49 ± 0.34	170 ± 9	47 ± 7	15 ± 1	1:25:32 ± 0:03:49	79.7 ± 3.4
FAST	3.51 ± 0.39	3.33 ± 0.46	169 ± 10	47 ± 7	15 ± 2	1:23:10 ± 0:05:41	77.8 ± 5.1
FAST-NA	3.56 ± 0.37	3.42 ± 0.36	169 ± 11	47 ± 8	15 ± 2	1:20:57 ± 0:08:08	79.1 ± 3.7

CFED, carbohydrate trial; CFED-NA, carbohydrate with nicotinic acid trial; FAST, fasted trial; FAST-NA, fasted with nicotinic acid trial. $\dot{V}O_2$, oxygen uptake; $\dot{V}CO_2$, carbon dioxide production; HR, heart rate; RR, respiratory rate; RPE, rate of perceived exertion; % $\dot{V}O_2$max, percentage of maximal oxygen uptake. Values are means ± SD.

4.5 Discussion

The novel finding of the present study was that the suppression of lipolysis and the exercise-induced increase in plasma FFA concentrations via NA ingestion did not impair half-marathon running capacity in competitive male athletes. Indeed, regardless of substrate priming by pre-event nutrition (a CHO-rich pre-race meal or following an overnight fast), intense exercise was CHO-dependent, with fat oxidation providing only a small contribution towards total energy expenditure. This is the first study to administer NA to well-trained runners to suppress blood-borne FA availability during high-intensity running.

A primary goal of the current investigation was to determine whether blunting the normal exercise-induced rise in plasma FFA would have a detrimental effect on the performance of an endurance running event in competitive athletes. Although time to fatigue protocols measure exercise capacity rather than performance *per se*, the protocol implemented in this study was necessary to allow steady-state measures of whole-body rates of substrate oxidation. The primary finding of no difference in the running distance covered between the four trials when running at ~80% $\dot{V}O_2max$ (Figure 4.2) supports the original hypothesis that fat oxidation plays only a minor role in endurance events lasting ~90 min when CHO availability is high. A step-wise reduction in the mean distance covered whereby CFED > CFED-NA > FAST > FAST-NA was observed, although such differences failed to reach statistical significance. Indeed, ES statistics revealed small to moderate reductions in performance when exercising fasted or fasted with NA compared to when CHO fed, respectively. The small decrement in distance covered measured in the overnight fasted trials in comparison to the CHO fed trials (6.6%) supports the importance of ingesting CHO in the hours prior to and during high-intensity running to increase CHO availability, turnover and oxidation rates and ultimately optimise

performance. Indeed, it has long been know that high CHO availability can delay the onset of fatigue during prolonged intense exercise (Coyle et al., 1986). Although a ~7% difference in the distance covered appears a worthwhile improvement for an athlete, it is important to note that the magnitude of the increase in distance covered in the trials in which pre-exercise CHO was consumed was well below the 10-15%, which has been estimated as a meaningful variation when using a time to volitional fatigue trial of similar exercise duration (Hopkins et al., 1999).

The majority of studies which have previously investigated the NA-induced suppression of fat availability on exercise performance have focused on cycling protocols (Bergstrom et al., 1969; Hawley, 2002; Jeacocke & Burke, 2010; Torrens et al., 2016). Torrens et al. (2016) reported no difference in cycling performance when participants completed a 90 min cycling TT (~300 W, 82% $\dot{V}O_2$max) following the ingestion of NA in a CHO-fed state in comparison to a control trial. Equally, no differences in cycling performance were observed during a ~30 min cycling TT (320 W, ~80% $\dot{V}O_2$max) or a 3.5-mile cycling TT (~12 min) when NA was ingested in a CHO-fed state in comparison to a control trial (Hawley et al., 2000; Murray et al., 1995). The findings of these studies might be considered predictable, based on the nature (short duration, high-intensity) or mode (cycling) of exercise, both of which favour high rates of CHO oxidation (Achten et al., 2003; Capostagno & Bosch, 2010; Romijn et al., 1993; van Loon et al., 2001). The current study adds to the body of knowledge by confirming the importance of CHO as a substrate for sporting activities at higher exercise intensities and during running, where it is recognised that rates of fat oxidation are higher at the same relative intensities than observed during cycling (Achten et al., 2003; Capostagno & Bosch, 2010). The half-marathon event was chosen for investigation because endogenous fat and CHO stores would be highly available as energy substrates under control conditions (Hawley & Burke, 1997) and thus a

change in performance and fuel use associated with a change in substrate availability would indicate the importance of this fuel source.

The second major finding of the current study was that participants were reliant on CHO substrates to fuel muscular work under all experimental conditions as indicated by the predominant contribution of CHO to total energy expenditure (83-91%, Figure 4.6).
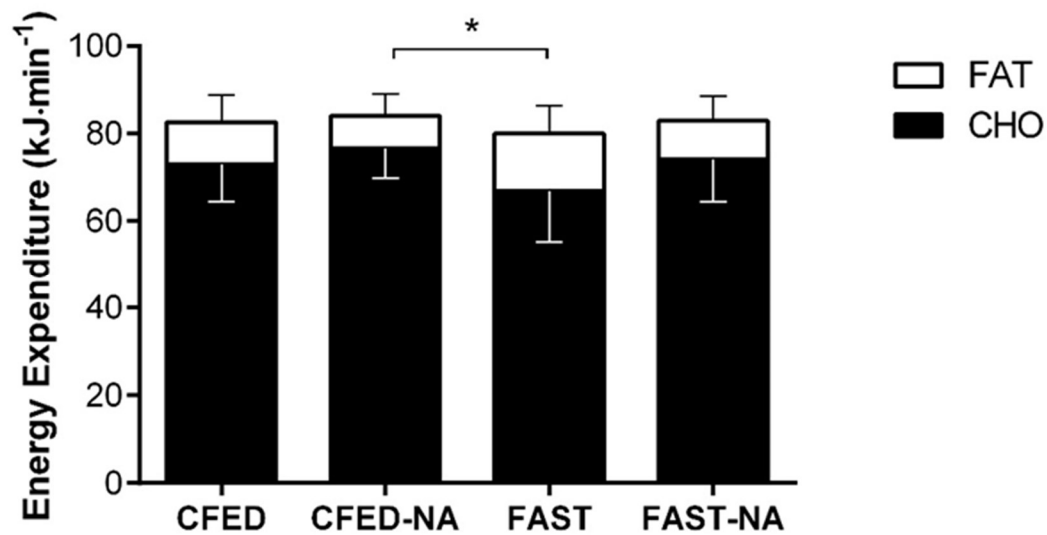
Figure 4.6. Estimated energy expenditure during half-marathon running for all experimental trials. CFED, carbohydrate trial; CFED-NA, carbohydrate with nicotinic acid trial; FAST, fasted trial; FAST-NA, fasted with nicotinic acid trial. Values are means ± SD. Significantly different between treatments ($P < 0.05$), *CFED-NA to FAST.

The mean rates of CHO oxidation for all four experimental conditions was ~4 $g \cdot min^{-1}$ which amounts to a total of ~350 g of CHO for the exercise task (Table 4.1). Such a value is

well within the 400-500 g of muscle glycogen stored from the CHO loading diet (8 g·kg·BM^{-1} CHO) consumed by the trained runners in the 24 h prior to the half-marathon protocol (Hawley & Burke, 1997). It is noted that the absolute rates of CHO oxidation in the present study are substantially higher than those reported by Lee et al. (2014) during a half-marathon in which CHO was consumed. However, the well-trained status and faster running speeds (~15 km·h^{-1} vs. 12.2 km·h^{-1}) of the participants along with the higher energy demand of exercise explains such differences. Greater amounts of CHO (~55 g) were oxidised in the trials involving pre-exercise CHO intake compared to overnight-fasted conditions; this is explained by greater CHO availability and the priming of the hormonal environment to increase rates of CHO utilisation (Coyle et al., 1985). The blunting of FFA availability with NA led to an equal increase in total CHO oxidation, regardless of pre-exercise CHO intake. However, even under conditions that should favour fat oxidation (overnight fasting, absence of exogenous CHO intake during exercise), CHO remained the predominant fuel source (83 % total energy expenditure).

It has long been known that the ingestion of NA alters fuel availability and hence muscle substrate selection during exercise (Bergstrom et al., 1969). A blunting of the typical exercise-induced rise in FFAs has been demonstrated in previous studies that have administered NA in cycling protocols (Hawley et al., 2000; Murray et al., 1995) and was clearly demonstrated in the present study, independent of CHO status (Figure 4.3). However, there was an additive effect of pre-exercise CHO and NA on fat metabolism during exercise, as evidenced by the reduction in plasma FFA and glycerol concentrations after 60 min and 80 min of running, respectively, compared to pre-exercise CHO feeding alone. These findings support the results of Murray et al. (1995) who reported higher circulating plasma FFAs during submaximal cycling (~70% $\dot{V}O_2$max) when ingesting CHO compared to the co-ingestion of CHO plus NA.

Although the administration of NA in the current study suppressed adipose tissue lipolysis as evidenced by the reduction in plasma FFAs, and likely the transport of FFA via cholesterol lowering effects which reduce hepatic synthesis of very-low density lipoprotein, total fat oxidation during the running protocol was estimated to be ~21 g with no difference observed between trials (Table 4.1). As there was only a small contribution from plasma FFAs to total fat utilised, it is likely that a large proportion of the fat oxidised was from IMTGs (Romijn et al., 1993; van Loon et al., 2001). Consequently, the small yet obligatory contribution of endogenous fat substrates when running at high intensity, irrespective of nutritional status pre-exercise should not go unrecognised.

Bergström et al. (1969) reported higher RQ values measured via arteriovenous oxygen difference across the working leg and thus greater CHO utilisation during submaximal cycling exercise following administration of NA compared to a control trial. The higher RQ was associated with a 33% increase in muscle glycogen utilisation, greater arterial blood lactate concentrations and a reduction in arterial FFA and glycerol concentrations. The measurement of whole body RER in the present study makes it difficult to isolate the energy contribution from individual CHO sources. However, NA ingestion was associated with a greater increase in blood lactate concentrations at the onset of exercise, regardless of the effect of pre-exercise CHO intake on lowering blood glucose concentrations (Figure 4.4). This provides indirect evidence for a greater reliance on endogenous CHO sources (i.e. muscle and liver glycogen) as previously reported (Bergstrom et al., 1969).

When investigating the interaction between training status, exercise intensity and pre-exercise nutritional state on substrate oxidation, Bergman and Brooks (1999) reported that substrate oxidation during graded cycling was largely determined by the relative intensity of

exercise. O'Brien et al. (1993) have also previously demonstrated the importance of exercise intensity during simulated marathon running in 'fast' (completion time $\leq$ 2 h, 43 min, 73% $\dot{V}O_2max$) or 'slow' (completion time $\leq$ 3 h, 30 min, 65% $\dot{V}O_2max$) runners. These workers reported RER values and energy contribution from CHO substrates were significantly higher throughout the marathon in the faster runners (0.99, ~85-90% vs. 0.90, ~60-70%), even under conditions in which rates of fat oxidation would be expected to be maximised (i.e., overnight fasted, no CHO feeding during exercise).

While a recent study has highlighted the importance of fat oxidation during high-intensity exercise (Hetlelid et al., 2015), the results of that investigation should be interpreted with caution. Hetlelid et al. (2015) reported that RER values during interval run training (6 x 4 min work bouts at ~90-94% of $\dot{V}O_2max$) were reduced in well trained compared to recreational runners (0.88 vs. 0.95, respectively). However, these workers failed to demonstrate steady-state conditions during exercise, or correct for bicarbonate kinetics, so it is not known if breath $\dot{V}O_2$, and $\dot{V}CO_2$ values accurately reflect tissue oxygen consumption and CO_2 production (Frayn, 1983). Furthermore, even if the rates of fat oxidation were valid in that study (Hetlelid et al., 2015), they would still only contribute a maximum of 38% of total energy expenditure in their well-trained runners (Hetlelid et al., 2015), demonstrating CHO rather than fat dependence. Results from the current study support the original findings of O'Brien et al. (1993), who reported CHO dependency in both CHO fed and overnight fasted conditions when running a half-marathon, and further reinforce the fact that when highly trained athletes compete in endurance events lasting up to 3 h, CHO-, not fat-based fuels are the predominant fuel for the working muscles and CHO, not fat availability becomes rate limiting for performance (Hawley & Leckey, 2015).

This study includes several limitations which could be considered for future work in this area. Firstly, muscle biopsies were not included, therefore it was not possible to directly measure changes in muscle glycogen utilisation which would provide a clearer indication of the endogenous substrates utilised during the half-marathon. Additionally, the runner's habitual daily energy intake and composition was not measured therefore average niacin intake could not be assessed. Future work in this area should include a series of race and training simulations with muscle biopsy analysis, to determine substrate use and therefore help practitioners provide appropriate nutritional advice for event and training session requirements.

In conclusion, the results of the current study show that well-trained runners are CHO dependent when running a half-marathon at race pace. Furthermore, when CHO availability is high, blunting the exercise-induced increase in FFA via NA ingestion did not impair intense exercise capacity in competitive athletes. During exercise of this intensity and duration, fat oxidation constitutes only a small percentage of overall energy expenditure independent of pre-event CHO status and CHO availability during exercise. While there is a small but obligatory use of fat-based fuels during intense endurance exercise lasting ~90 min, the oxidation of CHO-based fuels predominate. Therefore, endurance athletes should undertake dietary strategies that ensure high-CHO availability before and during competition to maximise rates of CHO oxidation and optimise race performance.

4.5.1 Funding

4.5.2 Acknowledgements

4.5.3 Disclosures

All authors report no conflict of interest

5. **Chapter 5- Ketone diester ingestion impairs time-trial performance in professional cyclists**

Publication statement:

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Leckey JJ, Ross ML, Quod M, Hawley JA, Burke LM. (2017). Ketone diester ingestion impairs time-trial performance in professional cyclists. *Front Physiol, 8,* 806. doi: 10.3389/fphys.2017.00806

5.1 Abstract

This study investigated the effect of pre- "race" ingestion of a 1,3-butanediol acetoacetate diester on blood ketone concentration, substrate metabolism and performance of a cycling time trial (TT) in professional cyclists. In a randomised cross-over design, 10 elite male cyclists completed a $\sim$ 31 km laboratory-based TT on a cycling ergometer programmed to simulate the 2017 World Road Cycling Championships course. Cyclists consumed a standardised meal (2 g·kg^{-1} BM CHO) the evening prior to a trial day and a CHO breakfast (2 g·kg^{-1} BM CHO) with 200 mg caffeine on the morning of a trial day. Cyclists were randomised to consume either the ketone diester (2 x 250 mg·kg^{-1}) or a placebo drink, followed immediately by 200 mL diet cola, given $\sim$ 30 min before and immediately prior to commencing a 20 min incremental warm-up. Blood samples were collected prior to and during the warm-up, pre- and post- TT and at regular intervals after the TT. Urine samples were collected pre- and post-warm-up, immediately post TT and 60 min post TT. Serum β- hydroxybutyrate, serum Acetoacetate and urine ketone concentrations increased from rest following ketone ingestion and were higher than placebo throughout the trial. Pre-exercise ingestion of the ketone diester resulted in a $2 \pm 1\%$ impairment in TT performance that was associated with gut discomfort and higher perception of effort. Ketone ingestion induces hyperketonemia in elite professional cyclists when in a CHO fed state, and impairs performance of a cycling TT lasting ~50 min.

5.2 Introduction

Substrate utilisation during exercise is influenced by several factors including the relative intensity and duration of exercise, an individual's training status and the effect of the preceding diet on both the substrate pool and the prevailing hormonal milieu (Hawley et al., 2015). As exercise intensity increases, there is a greater reliance on CHO based fuels (i.e., muscle and liver glycogen, blood glucose, lactate) and a reduction in the utilisation of fat substrates (Brooks & Mercier, 1994). Ketone bodies provide a potential source of readily oxidised fuel for skeletal muscle, but are predominately associated with conditions of metabolic stress such as starvation, where they are needed to preserve essential function of peripheral tissues including the brain and heart (Robinson & Williamson, 1980; Veech, 2004). However, there has been recent interest in the notion that increasing blood ketone concentrations could contribute to an enhancement of exercise performance by providing a readily available, alternative oxidative substrate for working muscle and "sparing" the limited stores of muscle glycogen (Pinckaers et al., 2017). As such, models of hyperketonemia from endogenous origin such as chronic exposure to a ketogenic diet (Burke et al., 2017) as well as introduction of exogenous sources of ketone bodies from ketone ester supplements (Cox et al., 2016) have been investigated. Nevertheless, there is some concern that the presence of high circulating concentrations of ketone bodies could inhibit the flux of other muscle substrates, either by impairing (rather than 'sparing') muscle CHO oxidation and/or inhibiting adipose tissue lipolysis (Evans et al., 2017). As such, the situations in which an available ketone supply may benefit exercise capacity or performance may be determined by the duration and/or intensity of exercise and the need for combinations of muscle substrate to meet the metabolic demands.

Ketone bodies, namely βHB, acetone and AcAc, are produced in the liver mitochondria from acetyl-CoA in response to an increased mobilisation of FFAs from adipose tissue in situations of reduced CHO availability (Robinson & Williamson, 1980). As summarised in recent reviews (Egan & D'Agostino, 2016; Pinckaers et al., 2017), under conditions of high CHO availability, circulating concentrations of ketone bodies are low, but are slightly elevated (0.1-0.5 mM) by an overnight fast and further raised by exercising in a fasted state (0.5-1.0 mM). Prolonged fasting/starvation (5 days) causes a maximal increase in rates of ketone body production (1-2 mmol·min^{-1} or 140-280 g·day^{-1}), leading to increased plasma concentrations that plateau under normal physiological conditions at ~7-10 mM. Meanwhile, chronic exposure to a ketogenic diet (low CHO [< 50 g·d^{-1}], low-moderate protein [~15% of energy], high fat [75-80% of energy]) raises plasma ketone bodies to 1-2 mM after several days, with concentrations reaching the apparent plateau achieved by prolonged fasting, according to the level of CHO restriction and duration of "keto-adaptation" (Pinckaers et al., 2017). Exogenous forms of ketone bodies include ketone salts, and more recently, ketone esters. Ingestion of the former appears to be less effective in increasing circulating ketone body concentrations and carries a significant salt load (Balasse & Ooms, 1968). Recently, a newly produced ketone monoester, R-3-hydroxybutyl R-3-hydroxybutyrate (Clarke et al., 2012), increases in plasma ketone concentrations (3-6 mM) within the hours following its ingestion (400-600 mg·kg^{-1} BM), although this may be altered by concomitant intake of food (Evans et al., 2017; Pinckaers et al., 2017).

To investigate the potential benefits to metabolism and sports performance, Cox and colleagues (2016) studied the effects of ingesting either CHO or CHO plus ketone ester (573 mg·kg^{-1} BM) on performance in trained cyclists. Their ingestion protocol induced a higher

blood D-βHB concentration during submaximal cycling (ranging between ~1.5-3 mM) and lead to a subsequent improvement in TT performance by ~2% following ketone ester and CHO ingestion compared to the ingestion of only CHO. However, aspects of the study design are inconsistent with conditions of "real world" cycling competition. Accordingly, this study examined the effect of pre-race ingestion of a ketone diester on blood ketone body concentrations, substrate metabolism and performance under conditions of elite professional cycling; ingestion of a pre-race CHO-rich meal, inclusion of a warm-up, involvement of world-class cyclists and simulation of a real-life course. It was hypothesised that this protocol would result in acute nutritional ketosis but that no performance improvement would be observed due to the high intensity nature of a real-life TT event which is dependent on the high rates of energy production from the oxidation of CHO-based fuels.

5.3 Materials and Methods

In keeping with the Australian Catholic University guidelines, the methods for this study are also presented in chapter 3. The methods below are formatted for *Frontiers in Physiology.*

5.3.1   Ethical Approval

This study conformed to the standards set by the *Declaration of Helsinki* and was approved (#20161005) by the Ethics Committee of the Australian Institute of Sport (AIS). After comprehensive details of the study protocol were explained to the participants verbally and in writing, all participants provided written informed consent.

5.3.2   Overview of study design

The study was a randomised crossover, double-blind, placebo-controlled design using elite (professional) cyclists attending a pre-season camp at the AIS, Canberra. On two separate

occasions, three days apart, participants completed a 20-min standardised warm-up and rested for 5 min prior to completing a 31 km TT performed on a cycling ergometer (Figure 5.1). Participants were randomised to consume a 1,3-butanediol acetoacetate diester (described subsequently) (KET) (two doses of 250 mg·kg^{-1} BM) or a viscosity and color-matched (PLAC) drink, given ~30 min before and immediately prior to commencing the warm up. It was not possible to completely replicate the taste of the KET drink, but a comparably novel and bitter-tasting PLAC was prepared from a mixture of flavor essences (rum, almond and bitters Angostura). Pilot testing revealed that the intake of small volume of diet cola immediately after the KET and *P*LAC was able to quickly mask the taste and texture of the previous drink. In any case, none of the participants had previously ingested a ketone ester supplement and were therefore unable to recognise its characteristics. In recognition of the World Anti-Doping Code under which these cyclists compete, it was ascertained that ketone supplements are not considered a prohibited substance by the World Anti-Doping Agency.

5.3.3 Participants

Eleven internationally competitive male cyclists (age, 25 ± 7 (SD) y; BM, 73.7 ± 7.6 kg; $\dot{V}O_2$peak, 71.4 ± 5.6 mL·kg·min^{-1}, 5.3 ± 0.3 L·min^{-1}; Maximal Aerobic Power (MAP), 494 ± 20 W) from the ORICA-BikeExchange UCI World Tour (Road Cycling) team participated in this study. Participants included world class elite (n = 8) (e.g. 2016 Paris-Roubaix winner, stage medalists from Tour de France, Tour Down Under, Giro d'Italia, Vuelta a España and Australian National championship Time Trial medalists) and highly trained under 23 riders contracted to the team (n = 3).
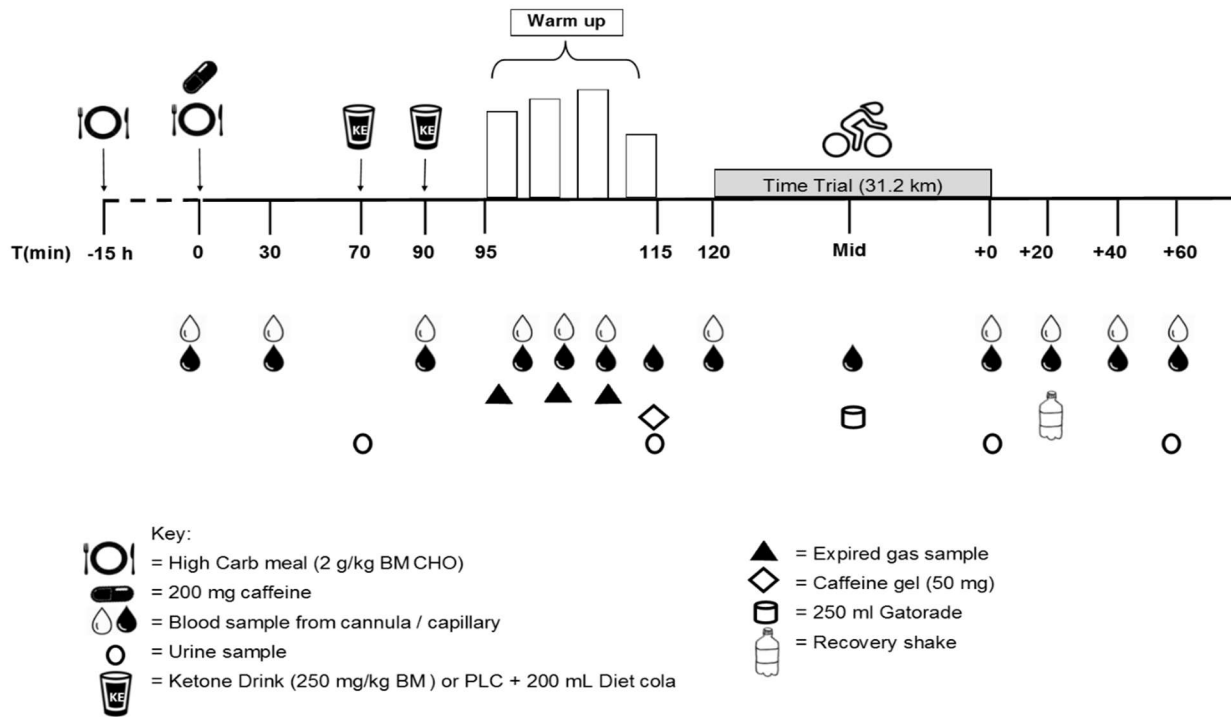
Figure 5.1. Overview of study design.

5.3.4 Preliminary Testing and familiarisation

Three days before commencing the experimental phase, participants visited the laboratory and completed an incremental exercise test and familiarisation with the cycling ergometer (Velotron, Racermate Inc., Seattle, WA, USA) and the experimental exercise protocol (simulated 2017 World Championships time trial course, Bergen Norway).

5.3.5 Incremental cycling test

Participants completed a 5-min warm-up at 150 W on the cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands), thereafter the test protocol started at 180 W and increased 30 W every 60 s until volitional exhaustion. MAP was determined as the power output of the highest stage completed. If the participant finished partway through a 60 s stage, MAP was calculated in a pro-rata manner. During the maximal test, expired gases were collected into a calibrated and customised Douglas bag gas analysis system, as previously described (Russell et al., 2002). $\dot{V}O_2$peak was calculated as the highest oxygen consumption recorded over a 30-s period. $\dot{V}O_2$peak and MAP were used to calculate the power output for the individualised warm up on subsequent trial days, described below.

5.3.6 Dietary control

CHO and caffeine intake were standardised the evening before and morning of a trial day and participants were also instructed to abstain from alcohol during the 24 h period prior to a trial day. Participants consumed an evening meal, snack and breakfast prepared by the team chef, providing a CHO content of 2 $g \cdot kg^{-1}$, 1 $g \cdot kg^{-1}$ and 2 $g \cdot kg^{-1}$ BM, respectively. Participants were also provided with a post-exercise recovery drink, 20 min after the completion of the time trial (1 $g \cdot kg^{-1}$ BM CHO and 25 g protein). The composition and timing of all meals was repeated prior to trial two.

5.3.7 *Synthesis of ketone ester*

The ketone ester synthesised, 1,3-butanediol acetoacetate diester, is a non-ionised sodium-free and pH-neutral precursor of AcAc (D'Agostino, 2012). The ketone ester was synthesised by transesterification of *t*-butylacetoacetate with *R,S*-1,3-butanediol (Savind, Seymour, IL, USA). The resultant product consisted of a mixture of monoesters and diester, the ratio of which could be adjusted by varying the stoichiometry of reactants. After synthesis the crude product was distilled under reduced pressure to remove all solvents and starting materials and the resultant ketone ester was obtained and assessed for purity using gas chromatography-mass spectrometry (GC-MS). All ingredients in this ketone drink are approved by the Food and Drug administration agency, USA.

5.4 Trial day procedure

5.4.1 *Participant preparation*

On each of the trial days, participants reported to the laboratory in a rested and overnight (10 h) fasted state, with the timetable creating a ~30 min time between individuals that was repeated on the subsequent trial day. The trial day protocol commenced with the placement of an indwelling cannula (22G; Terumo, Tokyo, Japan) into a cephalic vein while lying in a supine position to allow for repeat blood sampling. A fingertip sample of capillary blood was collected concomitantly with each cannula sample throughout each trial for immediate measurement of blood ketones (ß-hydroxybutyrate; FreeStyleOptium Neo, Abbott Diabetes Care, Doncaster, Australia). Following a resting blood sample (t = 0 min), participants were provided their standardised CHO breakfast including 200 mg caffeine (NO-DOZ Awakeners, Key Pharmaceuticals Pty Ltd, Macquarie Park, Australia), to mimic typical race preparations. Participants were instructed to consume the breakfast within 10 min, with a second blood

sample being collected at t = 30 min. At t = 70 min, participants provided a urine sample, were weighed and fitted with a heart rate monitor. At this time, they ingested the first dose (250 mg·kg^{-1} BM) of KET or PLAC in liquid form, followed immediately by 200 mL diet cola. At t = 90 min participants were seated on the Velotron ergometer, blood samples (4 mL) were collected and participants consumed their second dose in the same manner.

5.4.2   Warm up protocol

Following the second KET or PLAC drink, participants completed a standardised 20-min warm up on the cycle ergometer. The set-up of the bike was performed by team mechanics to replicate each rider's unique bicycle position and was fitted with a calibrated (Gardner et al., 2004) SRM cycling power meter (scientific version, 8 strain gauge, Schoberer Rad Meβtechnik; Jülich, Germany), set to sample at 1-s intervals. The warm up consisted of 3 x 5 min at 50% ventilatory threshold (VT), VT1 and VT1 plus 50% of the difference between VT1 and VT2 (156 ± 14, 312 ± 28, 355 ± 29 W, respectively), followed by 5 min self-paced cycling. Venous and capillary blood samples were collected every 5 min and expired gas was collected continuously during the first 15 min of the warm up. Immediately following the warm up participants, provided a urine sample and ingested an energy gel containing 50 mg caffeine (27 g CHO, PowerBarPowerGel, Nestle, Vevey, Switzerland). During this time (5 min), participants were free to complete their own preparations during which pre-TT blood samples were collected, participants were provided with standard pre-race instructions and the zero offset of the SRM crank was set according to manufacturer's instructions.

5.4.3 Cycling Time-Trial (2017 World Championship Road Cycling Time Trial simulation)

The TT consisted of a simulation of the 2017 Bergen World Championship TT course, based on global positioning system (GPS) mapping data (road altitude and distance) collected by the Orica cycling team staff (M. Quod, unpublished observations). Cyclists completed the 31.17 km TT as fast as possible and during the TT the only feedback provided to the participant was the distance covered (km), cycling gear-ratio (12–27/48-54) and road gradient (%). Participants were only informed of their TT results following the completion of both trials. Heart rate was collected every 5 km and RPE using the Borg 6 to 20 scale and capillary blood samples were collected at 15.74 km and immediately post TT. Participants ingested 250 mL of commercially available 6% CHO drink (Gatorade, IL, USA) at 15.74 km, as this distance corresponded to the point identified by the cyclists as the most appropriate opportunity to drink on the actual course. Samples of venous and capillary blood, and urine, were collected immediately following the TT and participants were weighed. At t = TT + 20 min, blood samples were collected and participants consumed a recovery drink (2 g·kg^{-1} BM CHO) and continued to rest quietly for a further 40 min. Blood samples were collected at t = TT + 40 min and t = TT + 60 min, with a final urine sample being collected at t = TT + 60 min. Following the removal of the cannula, participants participated in a semi-structured interview with a single researcher using a series of standard questions to probe perceived effort, motivation and comfort rating during the TT. When symptoms (e.g. gut discomfort and problems) were identified, a standardised Likert scale was used to quantify them into mild, moderate or severe. On completion of the second trial, participants were asked whether they could identify the trial in which they received the ketone ester, and the trial in which they had performed best. The

interview technique was used to probe levels of interest in using a ketone ester supplement in real competition.

5.4.4 Analytical Procedures

Capillary blood samples were analysed for concentration of ketones and lactate (Lactate Pro 2, Akray, Japan). Venous blood samples were collected into 4 mL SST vacutainers with immediate analysis of a small aliquot for blood glucose concentrations (Cobas Integra 400 plus, Roche Diagnostics, Switzerland). This venous sample was then centrifuged at 1,500 g for 10 min at 4 °C, and aliquots of serum were stored at -80 °C for later analysis. Samples were analysed for FFA concentrations using a non-esterified-fatty acid (NEFA) assay kit (Wako Pure Chemical Industries, Ltd, Osaka, Japan), βeta-hydroxybutyrate concentrations using a β-hydroxybutyrate assay kit (Sigma-Aldrich, Ltd, Australia) and AcAc concentrations using an acetoacetate assay kit (Abcam, Cambridge, UK), as per the manufacturer's instructions. Urine samples were analysed for urine ketones (namely AcAc) using ketone reagent strips (Keto-Diastix, Bayer, Germany).

5.4.5 Data Analysis

Statistical analysis was completed using SPSS (version 20 for Windows; SPSS, Chicago, IL). Paired t-tests were used to analyse average PO, cadence, heart rate and change in BM in the TT. Blood, urine, PO, heart rate, cadence, RPE and respiratory data from the two trials were analysed using a linear mixed model (treatment x time) (n=10) with the exception of respiratory data which includes (n = 9). When analysing respiratory gases, an RER > 1.0 was excluded from analysis as participants were not considered to be in steady state (n = 1, stage 3 for KET and PLAC). Statistical significance was set at $P < 0.05$ and data is presented as mean ± SD. TT performance was also analysed for magnitude-based ES between conditions using a custom

spreadsheet (Hopkins, 2006). Data were log-transformed to account for non-uniformity and ES with 90% confidence intervals (ES ± 90% CI) were calculated and classified as either trivial (-0.2 to 0.2 ES) small (0.2– 0.6, ES), moderate (0.6 –1.2 ES), or large (1.2–2.0 ES). Where the 90% CI overlapped small positive (0.2) and negative (0.2) values, the effect was considered to be unclear.

5.5 Results

5.5.1 Participant experiences

Eleven cyclists commenced this study, but one participant experienced such severe side effects from KET ingestion during and after the warm-up, including prolonged vomiting and dizziness that he was unable to complete the TT. This participant withdrew from further participation in the study. However, data for this participant have been provided in the following analysis in comparison to those of the other riders to investigate a possible explanation for the occurrence of these side effects. All participants reported gastrointestinal discomfort associated with the intake of the ketone diester. Symptoms ranged from major (dry retching and nausea; n = 2), to moderate nausea (n = 5) or moderate reflux (n = 1), and minor discomfort (mild nausea; n = 2). No similar symptoms were reported with the PLAC trial. All participants correctly nominated the trial in which they received KET, identifying it via the gastrointestinal side-effects. However, only four of the cyclists correctly identified the trial in which they completed the TT in the fastest time, with one cyclist equivocal. Although each of the riders nominated their gut symptoms as a distraction or interference to performance, six participants identified an "unusual" centrally-derived feeling during the TT in the KET trial that they thought might be associated with better performance. When asked if they would use the current KET supplement in actual competition, prior to the unmasking of performance results, only one participant (who

reported the least degree of discomfort during his KET trial) nominated being "possibly" interested. The remaining participants identified the need to remove the potential for illness and gut upset as well as to be sure of a robust performance effect before KET would be of value; "racing is hard enough without adding this complication".

5.5.2 Performance

All cyclists completed the TT in a faster time in PLAC compared with KET, with the crossover allocation of treatments meaning that there was no order effect on performance. Figure 5.2 displays the results of the cycling TT including group mean and individual performances. There was an impairment to overall performance time with KET ($2 \pm 1\%$, 58.2 s; small ES -0.42 ± 0.1, $P < 0.001$). There was an impairment in cycling performance time in the first segment of the course (0-18 km) ($P < 0.001$) and second segment with the climb included (18 km to 32 km) ($P = 0.004$) with KET ingestion compared to PLAC. Overall the KET condition was associated with a 3.7% reduction in average PO (KET 339 ± 37 W vs. PLAC 352 ± 35 W, $P < 0.001$, Figure 5.2B) and a lower cadence (KET 93 ± 6 rpm, PLAC 95 ± 6 rpm, $P = 0.06$, Figure 5.3C) compared to PLAC. There was an effect of time ($P < 0.001$) for power output and cadence during the TT, as displayed in Figure 5.3. A time × treatment interaction was reported for heart rate ($P = 0.001$) and average heart rate was significantly lower in the KET compared to PLAC condition (163 ± 7 vs 167 ± 9 bpm, respectively, $P < 0.01$) (Figure 5.3D). RPE increased in both the KET (16 ± 2 to 19 ± 2) and the PLAC (15 ± 2 to 19 ± 1) trials from mid- to post-TT ($P < 0.001$). There was no difference in RPE between trials despite the lower heart rate and PO in the KET condition.
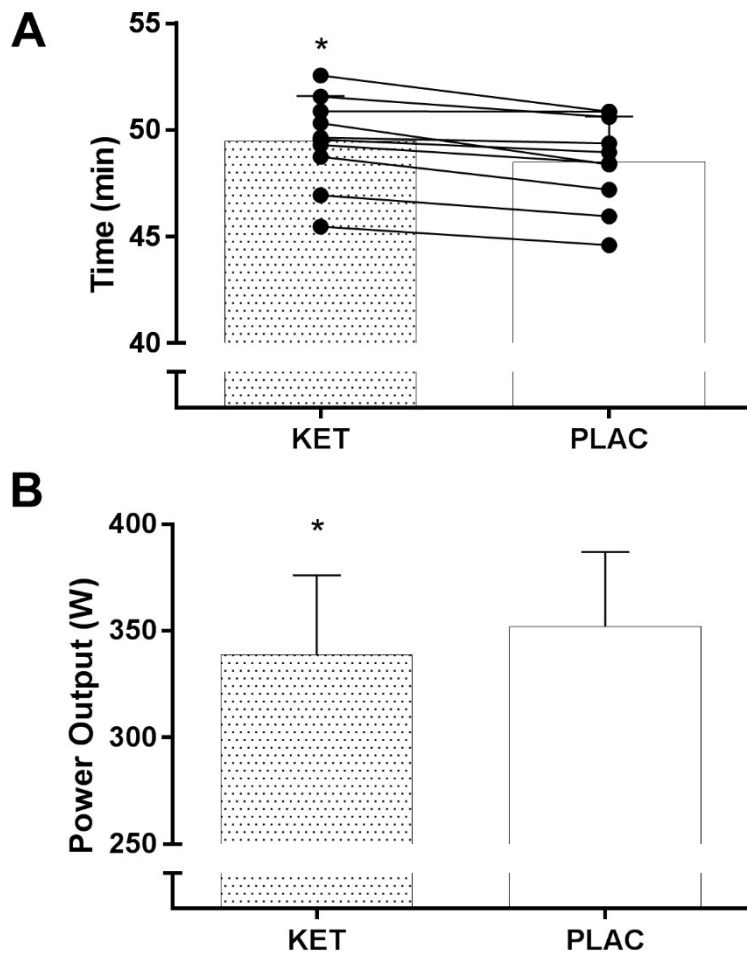
Figure 5.2. Mean and individual cycling TT performance time and mean power output following exogenous KET or PLAC ingestion. KET, ketone drink; PLAC, placebo drink. Values are mean ± SD. * KET different to PLAC.
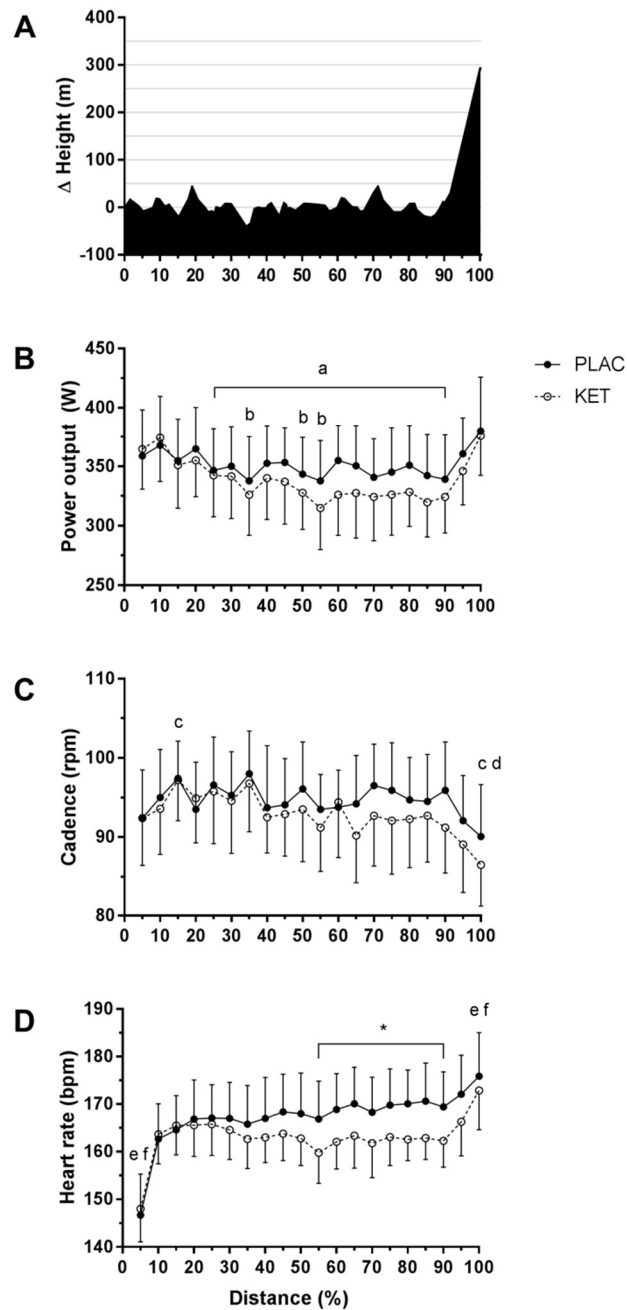
Figure 5.3. Course profile of World Championships time trial course (Bergen, Norway) (A), average power output (B), cadence (C) and heart rate (D) during time trial as a percentage of total distance, following exogenous KET or PLAC ingestion. Values are mean ± SD. a different to 5% in KET; b different to 5% in PLAC; c different to 10% in KET; d different to 10% in KET; * KET different to PLAC, e different to all other time points in KET, 50% different to f.

5.5.3 Serum metabolites

There was an effect of time ($P < 0.001$) and treatment ($P = 0.021$) for serum FFA concentrations (Figure 5.4A). FFA concentrations reduced from $t = 0$ during the 90 min following the CHO breakfast in both KET and PLAC trials (0.37 ± 0.10 mM to 0.27 ± 0.04 mM, $P < 0.02$). FFA concentrations were higher in PLAC vs. KET from pre- to post-TT ($P < 0.05$). A condition × time interaction was reported for serum βHB concentrations ($P < 0.001$) (Figure 5.4B). There was an increase in βHB concentrations in the KET trial following dose one of KET ingestion ($t = 90$ min) and βHB remained significantly higher than PLAC trial until $t = TT + 60$ min. An increase in βHB concentrations from $t = 0$ was measured in the PLAC trial at the onset of the warm up ($t = 100$ min) (Figure 5.4B) however βHB remained lower than in the KET trial. Serum AcAc concentration significantly increased from $t = 0$ min following dose one of KET ingestion ($P = 0.001$) and remained higher until $t = TT + 60$ min (Figure 5.4C).

5.5.4 Capillary blood and urine metabolites

There was a condition × time interaction for both urine ketone and capillary blood βHB concentrations ($P < 0.001$) (Figure 5.5A and B). No differences were observed at $t = 0$ between KET and PLAC for urine ketone concentration, but following KET ingestion, urine ketones were higher at pre-TT, post-TT and at $t = TT + 60$ min for KET. Blood βHB concentrations increased following the first dose of KET, compared with PLAC ingestion (0.32 ± 0.16 mM, $P = 0.001$). Blood βHB concentrations increased from pre-TT to post-TT in the KET trial ($P < 0.001$) and this increase was maintained until $t = TT + 60$ min ($P = 0.03$) (Figure 5.5B).

There was a significant condition × time interaction for blood glucose concentration ($P = 0.036$) and lactate concentrations ($P < 0.001$) (Figure 5.6A and B). Blood glucose concentrations were lower in KET following the first dose of KET ingestion, pre-TT and $t = TT + 40$, compared to

PLAC. Blood lactate concentrations increased from pre-warm up at the end of stage 3 (t = 110

min) in both the KET and PLAC but had returned to resting values pre- TT for both trials. Post-

TT, blood lactate concentrations were significantly lower in the KET trial compared to the

PLAC trial (8.6 ± 3.2 vs 13.1 ± 4.3 mM, P < 0.001, respectively).

5.5.5 *Respiratory parameters and body mass*

There was a main effect of time in the KET and PLAC trials for $\dot{V}O_2$, $\dot{V}CO_2$ and RER

(P < 0.001), where an increase was observed throughout the incremental warm up from stage 1

to stage 3 (Table 5.1). There was a main effect of time for BM (P < 0.001) where a similar loss

was measured in the KET (1.6 ± 0.7 kg) and PLAC (1.4 ± 0.4 kg) trials.

Table 5.1. Metabolic measures collected during 3 stage incremental warm up following exogenous KET or PLAC ingestion.

Variable	Treatment	Stage 1	Stage 2	Stage 3
$\dot{V}O_2$ (L/min)	KET	2.27 ± 0.27	3.95 ± 0.37*	4.42 ± 0.37
	PLAC	2.33 ± 0.26	4.01 ± 0.38*	4.45 ± 0.42
$\dot{V}CO_2$ (L/min)	KET	1.94 ± 0.21	3.66 ± 0.37*	4.28 ± 0.38
	PLAC	2.02 ± 0.24	3.70 ± 0.28*	4.31 ± 0.43
RER	KET	0.86 ± 0.04	0.93 ± 0.03*	0.96 ± 0.02
	PLAC	0.87 ± 0.03	0.93 ± 0.04*	0.96 ± 0.03

Values are mean ± SD. * different to stage 1 and stage 3.
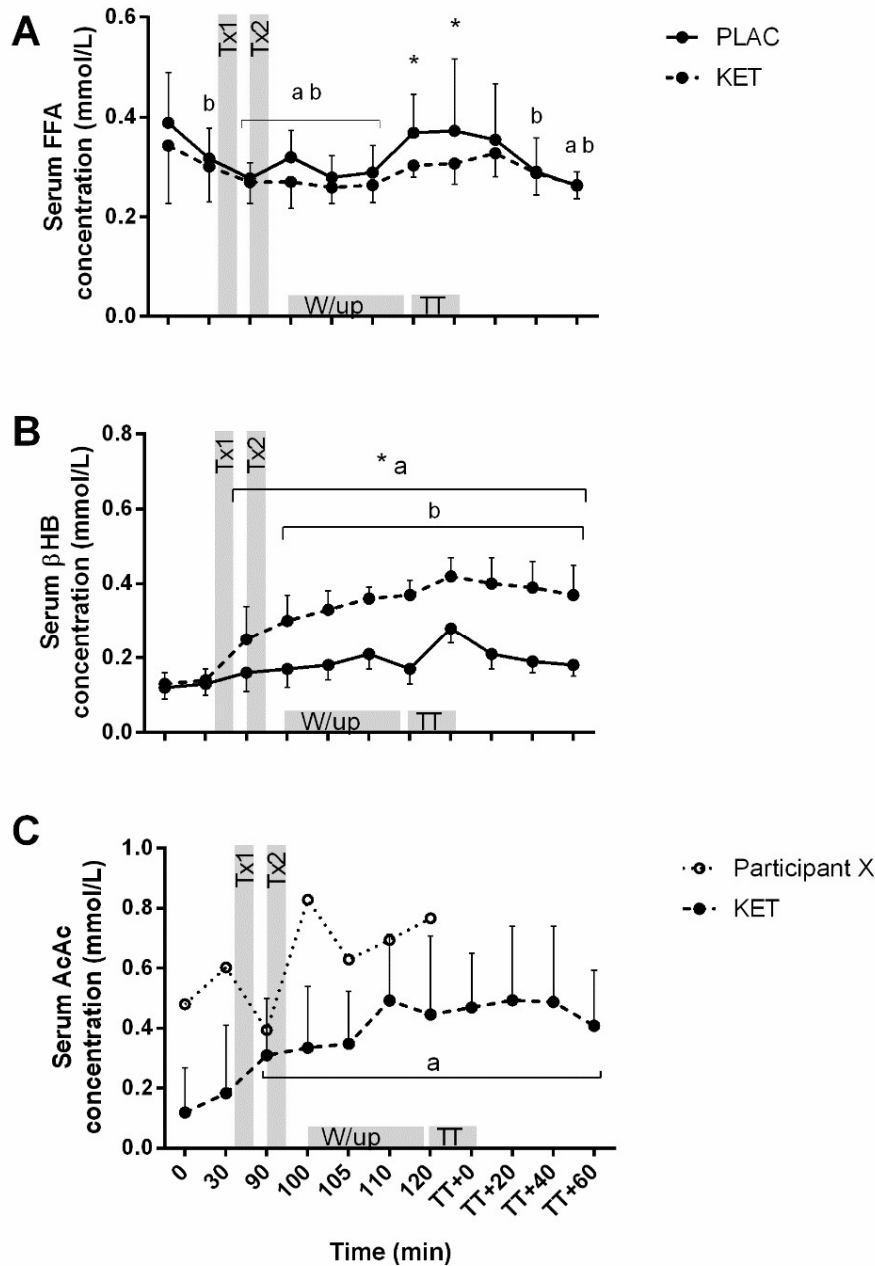
Figure 5.4. Serum FFA (A), βHB (B) and AcAc (C) concentrations following exogenous KET or PLAC ingestion while completing a cycling TT. Values are mean ± SD. Tx1 and Tx2 refer to dose one and two of ketone (KET) or placebo (PLAC) drink. FFA, free fatty acids; βHB, β-hydroxybutyrate; AcAc, acetoacetate. * KET different to PLAC at time point; a different to t = 0 min within KET; b different to t = 0 min within PLAC.
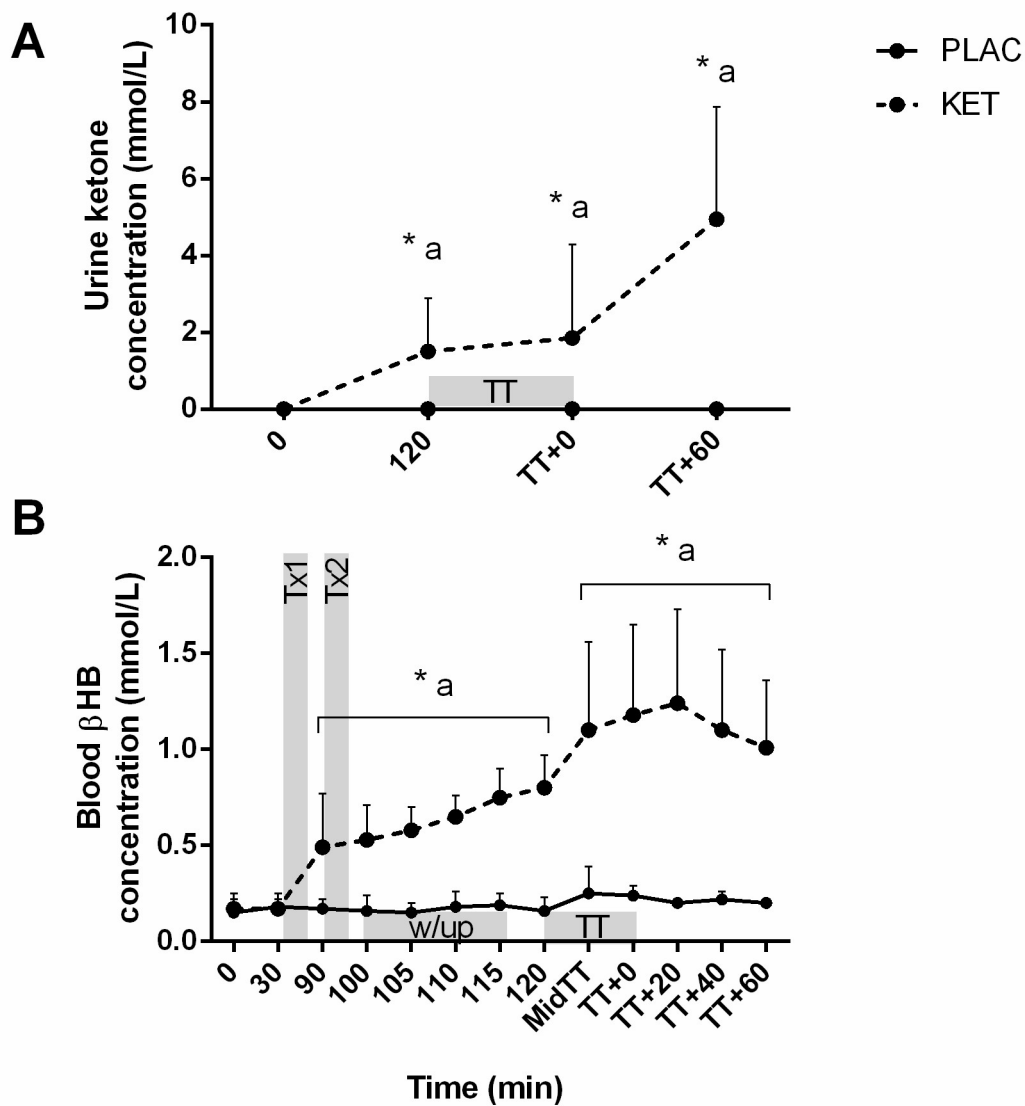
Figure 5.5. Urine ketone (A) and blood βHB (B) concentrations following exogenous KET or PLAC ingestion while completing a cycling time trial. Values are mean ± SD. Tx1 and Tx2 refer to dose one and two of ketone (KET) or placebo (PLAC) drink; w/up refers to warm-up; βHB, βeta-hydroxybutyrate. * KET different to PLAC at time point; a different to t = 0 min within KET; b different to t = 0 min within PLAC.
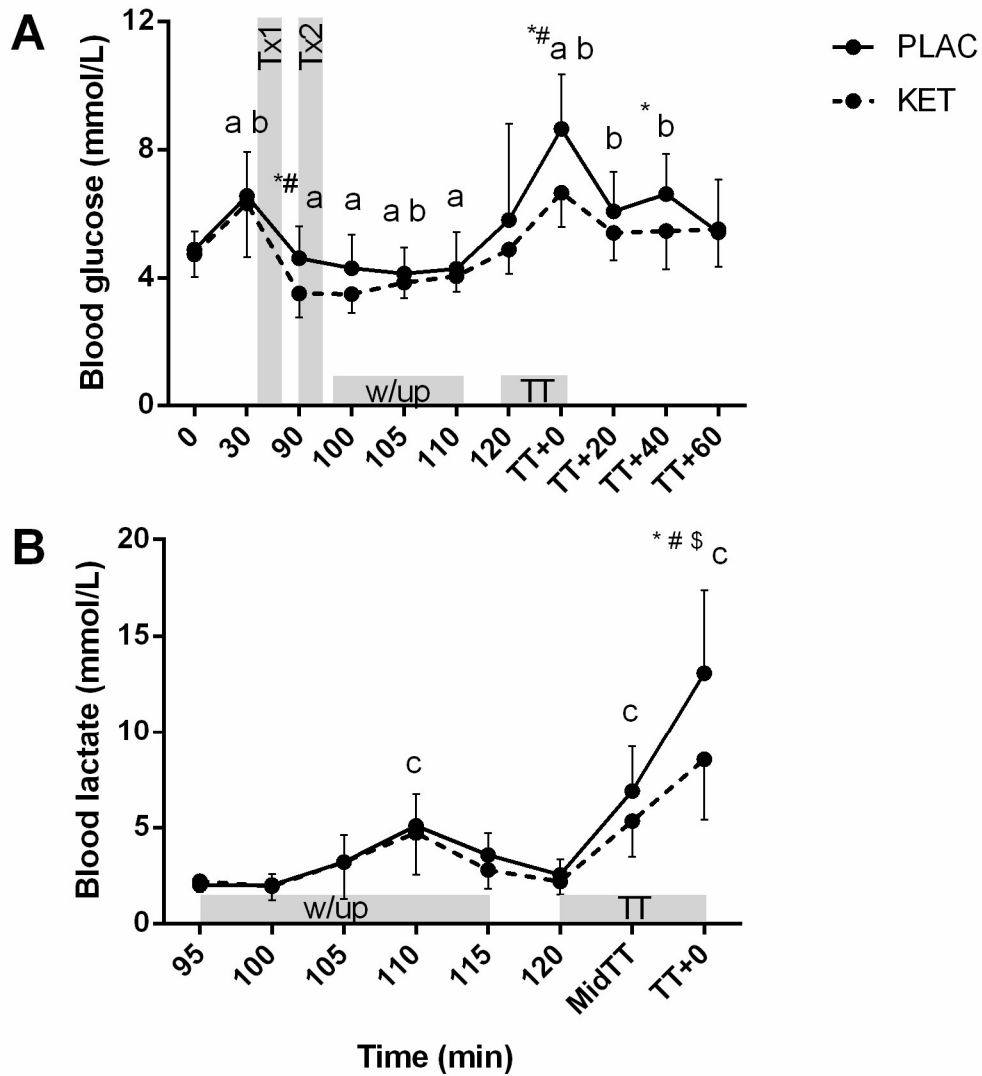
Figure 5.6. Blood glucose (A) and lactate (B) concentrations following exogenous KET or PLAC ingestion while completing a cycling time trial. Values are mean ± SD. Tx1 and Tx2 refer to dose one and two of KET or PLAC drink; w/up refers to warm-up. * KET different to PLAC at time point; a different to t = 120 min within KET; b different to t = 120 min within PLAC; c different to 95 min within and PLAC; # different to all other time points within PLAC; $ different to all other time points within KET.

5.6 Discussion

This is the first study to report the effect of pre-exercise supplementation with a ketone diester on the performance of a cycling TT under conditions simulating real-life competition: laboratory simulation of a World Championship TT course in world-class male road cyclists who undertook nutritional strategies mimicking competition practices with respect to CHO and caffeine supplementation. Although the protocol in the current study achieved hyperketonemia, as evidenced by increases in serum βHB and AcAc concentrations, there was an impairment of TT cycling performance in these elite cyclists. This outcome appears to be linked to the general observation of gut discomfort and intolerance among the study participants, with symptoms ranging from mild to severe. Results from this investigation add important information to sports nutrition, by adding a real-world element.

The primary aim of the present study was to address recent reports of enhanced sports performance associated with an acute increase in blood ketone concentrations following the intake of a ketone ester drink (Cox et al., 2016), by re-examining this concept in a more ecologically valid protocol. The investigation was undertaken as a collaborative project with a World Tour professional team of the International Cycling Union (UCI), offering an opportunity for them to make an evidence-based decision regarding the potential use of a highly discussed performance aid (Abraham, 2015). A number of features were included in the study design to optimise the reliability and validity of data, including opportunities to mimic the conditions under which the performance aid (ketone ester supplement) would be used. Team sports scientists were able to provide a simulation of the profile of the 2017 World Championships TT course (Bergen, Norway) on a cycle ergometer. Furthermore, the world-class cyclists from the team who participated in the study were personally motivated to receive individual and group

results and were highly experienced in TT cycling as well as familiarised to the specific laboratory-based course simulation. Race nutrition strategies (24 hours prior, pre-race and during race) were standardised and made realistic by involving meal preparation by the team chef to suit both sports nutrition guidelines (Thomas et al., 2016) and the cultural practices of the riders. This included attention to achieve adequate CHO availability in pre-race meals (as opposed to the overnight fasted state involved in other studies such as (Balasse, 1979; Cox et al., 2016; Fery & Balasse, 1986), as well as the real-life intake of caffeine and CHO supplements during the pre-race and within-race practices.

The primary finding of a $2.0 \pm 1\%$ (58 s) longer time to completion in the TT following ketone ingestion supports the initial hypothesis that ketone ingestion would not enhance TT performance (Figure 5.2). Although the gut disturbances were the likely cause of the performance impairment, it is noted that none of the cyclists achieved a faster time in the ketone trial, even when they reported very minor symptoms. Furthermore, the RPE in the ketone trial were similar to those on the placebo trial despite a lower power output and heart rate. This suggests that the gastrointestinal discomfort and/or some direct effect of ketones on the brain increased the perception of effort, and in accordance with the psychobiological model of pacing (Pageaux et al., 2014), the highly experienced cyclists reduced their work output to enable the TT to be completed without premature exhaustion. This finding warrants further investigation to confirm and explore the mechanisms. In the meantime, it is noted that the outcome of impaired performance with ketone ingestion is in contrast to previous studies (Clarke, 2015; Cox et al., 2016). Indeed, Clarke et al. (2015) and Cox et al. (2016) reported 1 - 2% improvements in 30 min rowing performance and 30 min TT performance, respectively,

following ingestion of a similar ketone dose to the current study (573 vs 500 mg·kg^{-1} BM, respectively) in combination with CHO, compared to CHO alone.

Due to the lack of a commercial supply, it was not possible to obtain the ketone monoester supplement used in the study of Cox et al. (2016). However a diester that is currently being investigated as a potential treatment for seizures resulting from central nervous system oxygen toxicity was sourced (D'Agostino et al., 2013) and used in a similar dose to Cox et al. (2016). This diester contains a racemic mixture of βHB (i.e. contains both D- and L-enantiomers of the βHB) and has the ability to elevate both βHB and AcAc in a 1:1 ratio. The use of enzymatic analysis in the current study measures only the D- enantiomer which is the main circulating form of βHB and the most likely to have a direct effect on substrate metabolism and skeletal muscle responses (Yamada et al., 2010).

The ketone drink was provided the in two doses, with the first bolus ingested 70 min prior to the TT. Based on previous research, the aim was to reach peak βHB concentrations at approximately ~ 1 h following ingestion (i.e. prior to the TT) (Pinckaers et al., 2017). A modest increase in serum βHB concentrations in the ketone trial was measured, reaching > 0.3 mM following the warm-up, but surprisingly serum βHB concentrations peaked immediately following the TT (> 0.4 mM) (Figure 5.3B). The capillary whole blood samples analysed for βHB concentrations measured values 2- to 3-fold greater than the serum samples (Figure 5.4B). This variation in D-βHB concentrations via enzymatic analysis (serum) and whole blood is consistent with previous literature reporting a - 0.5 to 0.6 mM higher concentration with handheld monitors (Pineda & Cardoso, 2015). This variation in measuring blood ketones in a controlled laboratory setting highlights the challenges athletes face in the field when aiming to reach and stay within the 'optimal' range for a performance benefit (Egan & D'Agostino, 2016).

A peak in serum AcAc concentrations was also measured following the warm-up, reaching ~ 0.5 mM (Figure 5.3C). Therefore when considering total circulating ketones measured (i.e. βHB and AcAc) and the L- enantiomer that has not been measured, it is likely that athletes would be in the 'optimal' range of 1 to 3 mM for a proposed performance benefit (Egan & D'Agostino, 2016). Although "nutritional ketosis" was achieved, βHB concentrations reported in the current study are much lower than those reported previously (Cox et al., 2016). Cox et al. (2016) reported an increased in βHB concentrations to ~ 2 mM within 20 min of ketone ester ingestion when co-ingested with CHO or ~ 4 mM when ingested alone. This variation in serum βHB is likely explained by a range of factors including the different ketone esters used, the elite training status of cyclists in the current study and the different pre-ingestion nutritional strategies were the current study focused on appropriate race preparation practices.

Although the current study has not measured the same increase in circulating ketone concentrations as Cox et al. (2016), ketones increased appropriately to alter metabolic responses compared to when a placebo was ingested. Blood glucose concentration was lowered in the ketone trial by ~ 1 mM within 30 min following ingestion of the first ketone dose, and following the TT blood glucose was ~ 2 mM lower than in the placebo trial. Additionally, the current study reported a 4.5 mM (35%) reduction in blood lactate concentration following the TT in the ketone trial compared to the placebo trial. These findings of reduced blood glucose and blood lactate concentrations are consistent with the data of Cox et al. (2016) during 60 min of exercise at 75 % maximal work load and following a 30 min TT, respectively. Lower circulating FFA concentration was also measured during the TT following ketone ingestion compared to a placebo. Participants were cycling at 340-350 W during the TT and thus estimated contribution of FFA oxidation to total energy expenditure would likely be low as at this intensity, the muscle

relies predominately on CHO-based fuels (Hawley & Leckey, 2015). However, the difference in FFA concentration between the ketone and placebo trials could be related to circulating ketone bodies having the ability to suppress lipolysis via inhibition of catecholamine's (Bjorntorp & Schersten, 1967).

As ketone bodies can be readily oxidised by skeletal muscle, expired gas was collected during the incremental warm-up. No differences in RER were measured between the ketone and placebo trials, although this could be related to the absolute exercise intensities attained by the elite subjects (155, 310, 355 W). Alternatively, the high ketone concentrations in the urine suggest that the ketones are not being oxidised by skeletal muscle. Estimated rates of substrate oxidation have not been estimated due to βHB and AcAc yielding respiratory exchange quotient values of 0.89 and 1.00, respectively and thus without appropriate correction factors for CO_2 displacement and urine volume this would lead to an inaccurate representation of substrate utilisation (Frayn, 1983; Pinckaers et al., 2017). A modest reduction in heart rate during the TT was also reported in the ketone trial compared to the placebo trial (5 bpm) which may be associated with a slightly reduced average power output and in the ketone trial.

Of the ten participants who completed the trials, all reported gastrointestinal discomfort associated with the intake of the ketone ester including dry retching, mild to moderate nausea, reflux and minor discomfort. Furthermore, one participant was unable to start the TT due to prolonged vomiting and dizziness. This participant also experienced the highest concentrations of serum AcAc concentration when compared to the other ten participants (Figure 5.4C, participant X); suggesting bioavailability of the ketone diester may impact individual responses following ketone ingestion. Although the side effects of ketone esters are not frequently discussed, Cox et al. (2015) has provided evidence that participants have experienced a range

of adverse effects including vomiting, nausea, diarrhea and abdominal pain. These side effects have been associated with high dose of ketone ester and the consumption of the ester with a milk-based drink (Clarke, 2015). While it is possible that different dosing strategies, or the use of a different ketone ester product might eliminate or greatly reduce the gut problems seen in the current study, it is unclear whether a performance enhancement could be expected with exogenous ketone use in sporting events undertaken under the conditions employed in the present study.

There are several limitations in the current study which could be considered for future research. Due to the short, high intensity nature of the time trial and the aim to replicate a race, the researchers were unable to collect expired gas during the cycling duration and estimate changes in substrate metabolism following the ketone ingestion. Additionally, the βHB concentrations in current study were not as high as previously reported which may have contributed to the different findings reported. Future research should consider study designs that truly replicate race-day conditions (i.e. following optimal CHO ingestion) including race nutrition supplementation (e.g. caffeine, sodium bicarbonate, beetroot jucie). Additionally, factors such as type of ketone ester, tolerability, timing and optimal dose should be considered in further work.

In conclusion, the results of the current study show that ingestion of a 1, 3 -butanediol acetoacetate diester under conditions of optimal race nutrition (i.e. CHO fed) results in increases in βHB and AcAc concentrations. The diester was associated with gut discomfort and intolerance among the cyclists with symptoms ranging from mild to severe. Despite optimal nutritional support (i.e. CHO breakfast, feeding during the TT and caffeine ingestion) for

performance, ketone ingestion was associated with an increase in perception of effort, leading to an impairment of TT performance in elite professional cyclists.

5.6.1 Funding

5.6.2 Acknowledgements

5.6.3 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be a potential conflict of interest.

6. Chapter 6- High dietary fat intake increases fat oxidation and impairs skeletal muscle mitochondrial respiration in trained humans

Publication statement:

This chapter is comprised of the following paper published in *the Federation of American Societies for Experimental Biology (FASEB).*

6.1 Abstract

High-fat, low-carbohydrate (CHO) diets increase whole-body rates of fat oxidation and down-regulate CHO metabolism. Substrate utilisation and skeletal muscle mitochondrial respiration were measured to determine if these adaptations are driven by high-fat or low-CHO availability. In a randomised crossover design, eight male cyclists consumed five days of a high-CHO diet (HCHO, > 70% energy intake (EI)), followed by five days of either an isoenergetic high-fat (HFAT, > 65% EI) or high-protein diet (HPRO, > 65% EI) with CHO intake 'clamped' at < 20% EI. During the intervention, participants undertook daily exercise training. On day six, participants consumed a high-CHO diet, prior to undertaking 100 min of submaximal steady state cycling plus a ~30 min time trial. Following five days of HFAT, skeletal muscle mitochondrial respiration supported by octanoylcarnitine and pyruvate as well as uncoupled respiration was decreased at rest, and rates of whole-body fat oxidation were higher during exercise compared to HPRO. Following one day of HCHO intake, mitochondrial respiration returned to baseline values in HFAT while rates of substrate oxidation returned towards baseline in both conditions. These findings demonstrate that high dietary fat rather than low-CHO intake contributes to reductions in mitochondrial respiration and increases in whole-body rates of fat oxidation following a high-fat, low-CHO diet.

6.2 Introduction

High-fat, low-carbohydrate (CHO) diets have increased in popularity over the past two decades with regards to their efficacy for improving both metabolic health profiles (Westman et al., 2008) and athletic performance (Burke et al., 2000; Burke et al., 2017). Short-term (1-3 week) ingestion of a high-fat, low-CHO diet when compared with an isoenergetic high-CHO diet for the same duration increases rates of whole-body and muscle fat utilisation and decreases the rate of muscle glycogenolysis during submaximal exercise (Burke et al., 2000; Carey et al., 2001; Stellingwerff et al., 2006; Zderic et al., 2004). Such metabolic perturbations are robust and persist in the face of high-CHO availability from both endogenous and exogenous sources (Burke et al., 2002; Carey et al., 2001; Havemann et al., 2006). Impaired glycogenolysis as a consequence of high-fat, low-CHO diets has been explained by decreased pyruvate dehydrogenase (PDH) activation (Stellingwerff et al., 2006), suggesting impaired metabolic flexibility in skeletal muscle. A range of alterations in the activities of regulatory enzymes and/or signaling proteins in the pathways underlying skeletal muscle fat and CHO metabolism are likely to explain the changes observed with adaptation to a high-fat diet. However, to date it has not been possible to determine whether such adaptations are driven by high-fat or low-CHO availability as the protocols used in previous studies involved changes to both macronutrients simultaneously (Burke et al., 2000; Burke et al., 2002; Carey et al., 2001; Stellingwerff et al., 2006). Therefore, in order to elucidate the underlying mechanisms driving changes in metabolic flexibility, high-fat dietary intake must be compared to an isoenergetic diet, where CHO intake is clamped in both dietary interventions. Few studies have determined changes to skeletal muscle in well-trained humans following a high-fat diet and to date, no study

has assessed mitochondrial respiration in this population to determine if this could explain changes in metabolic flexibility.

Therefore, the current investigation aimed to determine whether the metabolic perturbations induced by a high-fat diet are a result of high-fat or low-CHO availability. Well-trained humans were fed five days of either a high-fat diet or an isoenergetic high-protein diet (~65% energy intake (EI)) with CHO intake 'clamped' to < 20% of total daily EI (2.6 g·kg^{-1} body mass). Whole-body expired gas measures were utilised together with assessment of skeletal muscle substrates, mitochondrial respiration and signaling proteins with putative roles in substrate metabolism in an effort to identify mechanisms underlying changes in the patterns of substrate oxidation observed following a high-fat diet. It was hypothesised that whole body rates of fat oxidation would be greater following high-fat compared to a high-protein diet due to high-fat rather than low-CHO availability driving the shifts in fuel utilisation and skeletal muscle mitochondrial respiration.

6.3 Methods

In keeping with the Australian Catholic University guidelines, the methods for this study are also presented in chapter 3. The methods below are formatted for *the Federation of American Societies for Experimental Biology (FASEB)*.

6.3.1 Ethical Approval

This study conformed to the standards set by the *Declaration of Helsinki* and was approved by the Human Research Ethics Committee of Australian Catholic University and registered with the Australian New Zealand Clinical Trials Registry

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(ACTRN12616000433404). Participants completed a medical history questionnaire to ensure they were free from illness and injury before commencing the study and were informed of all experimental procedures and possible risks prior to providing their written, informed consent.

6.3.2 Overview of study design

Eight well-trained male cyclists with a history of endurance training and riding > 200 km·week^{-1} were recruited for this study. Participant characteristics were: age, 25 ± 4 (SD) y; body mass (BM), 77.3 ± 7.0 kg; $\dot{V}O_2$peak, 64.0 ± 3.5 mL·kg·min^{-1}; peak power output (PPO), 380 ± 36 W. An overview of the study design is shown in Figure 6.1. Each participant completed two experimental conditions in a block randomised, crossover design while undertaking supervised training. There was a ~14 day wash out period between conditions. It was not possible to blind participants to the dietary interventions. However, the principal researchers completing the data collection and performance measures were blinded to the order of experimental trials.

Lab trial
● Blood sample pre and post exercise
❙ Muscle biopsy pre and/or post exercise

Figure 6.1 An overview of the study design showing the five days high-CHO diet followed by five days high-fat or high-protein diet (D1 to D7) with one day of a high-CHO diet. CHO, carbohydrate; HIIT, high-intensity interval training; TT, time-trial.

6.3.3 Preliminary testing

Each participant completed an incremental test to volitional fatigue on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, Netherlands) to determine $\dot{V}O_2peak$ and PPO (Hawley & Noakes, 1992). During the maximal test and all subsequent experimental trials, expired gas was collected every 30 s via open-circuit spirometry (TrueOne 2400; Parvo Medics, Sandy, UT) and the instantaneous rates of O_2 consumption ($\dot{V}O_2$) and CO_2 production ($\dot{V}CO_2$) were used to calculate the respiratory exchange ratio (RER). Before each test, gas analysers were calibrated with commercially available gases (16% O_2, 4% CO_2) and volume flow was calibrated using a 3 L syringe. An individual's $\dot{V}O_2peak$ was determined as the highest 30-s average. These data were used to calculate the work rate corresponding to 63% and 80% of PPO for the two experimental rides.

6.3.4 Experimental trials

Participants followed a 'controlled' high-CHO diet (72% EI), 10 $g^.kg^{-1}$ BM [HCHO]) for five days prior to an experimental trial (see Table 1). Participants reported to the lab on the 5th day after an overnight fast and a resting blood sample (6 mL) was collected from an antecubital vein. Participants were then provided a standardised breakfast (2 $g^.kg^{-1}$ BM CHO). Two hours following breakfast, participants were weighed and a second blood sample was collected before they completed a 20 min continuous ride at 63% PPO. Expired gas and measures of heart rate (HR) and rating of perceived exertion (RPE) were collected during the last 5 min of the ride (Borg, 1973). Water was consumed *ad libitum* and upon completion of the ride, a third blood sample was collected prior to participants leaving the lab for the final (5th) day of the HCHO diet.

The following morning, participants reported to the lab overnight fasted and a cannula (22G; Terumo, Tokyo, Japan) was inserted into an antecubital vein and a resting blood sample (6 mL) was collected. A resting muscle biopsy was then taken from the vastus lateralis using the percutaneous biopsy technique with suction applied (Evans et al., 1982). Participants then repeated the 20 min continuous ride at 63% PPO in the fasted state, before commencing a high-intensity interval session (HIIT) (8 x 5 min at 80% PPO), as previously described (Stepto et al., 2001). The purpose of this interval session was to reduce muscle glycogen stores in both conditions prior to the dietary intervention.

6.3.5   Diet and training interventions

Participants commenced five days of either a high-fat (HFAT) or a high-protein (HPRO) diet. The HFAT and HPRO diets comprised 67% EI from fat or protein and 19% EI from CHO (Table 1). Protein was provided as an alternative macronutrient to meet energy requirements, while CHO was 'clamped'. Total EI was 0.22 $MJ \cdot kg^{-1}$ BM. The HFAT diet was comprised of ~55% saturated and 45% unsaturated (mono and polyunsaturated) fats. Fiber intake was matched for both diets. All meals, snacks and energy-containing fluids were provided to participants in pre-prepared packages, with diets individualised for food preference. Participants completed a daily food checklist to maximise compliance and recorded all fluid (water) consumed on a daily basis during both trials. Caffeine ingestion was not permitted 24 h prior to an experimental trial and participants refrained from alcohol during the intervention period. During this time, participants followed a prescribed training program described previously (Burke et al., 2000) that closely matched each individual's habitual road cycle training volume. Training was matched for each experimental treatment and participants were instructed to ride at a rating of perceived exertion (RPE) that corresponded to 11-13 (Borg, 1973) during each on-

road session. Participants reported to the lab on day 4 and completed the same HIIT session as on day 1. On the morning of day 6, participants reported to the lab in a fasted state and a resting blood sample (6 mL) and muscle biopsy were collected before they completed a 20 min ride at 63% PPO. Participants were then provided with 1 day of a high-CHO diet (10 g·kg^{-1} BM CHO) (Table 1).

6.3.6   *Performance ride*

After an overnight fast, participants reported to the laboratory to complete a performance ride consisting of 100 min steady state (100SS) cycling at 63% PPO, followed by a 7 kJ·kg^{-1} BM time trial (TT). On arrival at the laboratory, a cannula was inserted into an antecubital vein and a fasted blood sample (10 mL) was collected. A muscle biopsy was then taken 2-3 cm distal from the previous incision. Participants then consumed breakfast (2 g·kg^{-1} BM CHO) and rested for 120 min. Immediately prior to exercise participants were weighed and a second blood sample was collected. During exercise blood samples (10 mL) and measures of RPE and HR were collected every 20 min, with expired gas collected at 15, 35, 55, 75, and 95 min. Participants were provided with CHO in the form of isotonic gels (SiS GO Isotonic Gel; Blackburn, UK) and a 6% CHO solution (933 mL fluid, 2 gels total) every 20 min throughout the ride at a rate of 60 g·h^{-1} and water was consumed *ad libitum* during each trial. Immediately upon completion of the 100SS ride, a further muscle biopsy was taken. Participants then voided their bladder and had a 3 min rest prior to commencing the TT. Participants were instructed to complete the TT as fast as possible with visual feedback of cadence and verbal feedback of elapsed work as a percentage of the total work (every 10%). Participants were only provided the results of their TT performance upon study completion. Blood samples were collected immediately before and after the TT.

Table 6.1. Macronutrient content of the high-carbohydrate (HCHO), high-fat (HFAT) and high-protein (HPRO) diets consumed.

Total Energy Intake (TEI).

	Energy		Carbohydrate			Protein			Fat		
	MJ	MJ/kg	Total (g)	g/kg	% TEI	Total (g)	g/kg	%TEI	Total (g)	g/kg	% TEI
HCHO	17.0 ± 1.4	0.22 ± 0.0	769.4 ± 63.4	10.0 ± 0.1	73.0 ± 0.5	140.5 ± 11.8	1.8 ± 0.0	14.2 ± 0.4	58.2 ± 6.3	0.8 ± 0.0	12.8 ± 0.8
HFAT	17.0 ± 1.5	0.22 ± 0.0	196.1 ± 19.7	2.5 ± 0.1	18.3 ± 0.7	138.7 ± 10.7	1.8 ± 0.1	13.7 ± 0.4	315.5 ± 26.8	4.1 ± 0.1	68.0 ± 0.6
HPRO	17.0 ± 1.5	0.22 ± 0.0	200.3 ± 19.5	2.6 ± 0.1	18.5 ± 1.0	679.8 ± 66.7	8.8 ± 0.5	66.7 ± 0.9	69.3 ± 7.4	0.9 ± 0.1	14.8 ± 0.7

6.3.7 Rates of substrate oxidation and total energy expenditure

Whole body rates of CHO and fat oxidation ($g \cdot min^{-1}$) were calculated from respiratory gas samples collected during rides using the non-protein RER equations (Peronnet & Massicotte, 1991) which are based on the assumption that $V\dot{O}_2$ and $VC\dot{O}_2$ accurately reflect tissue O_2 consumption and CO_2 production:

CHO oxidation ($g \cdot min^{-1}$) = 4.585 $\dot{V}CO_2$ ($L \cdot min^{-1}$) - 3.226 $\dot{V}O_2$ ($L \cdot min^{-1}$)

Fat oxidation ($g \cdot min^{-1}$) = 1.695 $\dot{V}O_2$ ($L \cdot min^{-1}$) - 1.701 $\dot{V}CO_2$ ($L \cdot min^{-1}$).

Rates of CHO and fatty acid oxidation ($\mu mol \cdot kg \cdot min^{-1}$) were calculated by converting the rates of oxidation ($g \cdot kg \cdot min^{-1}$) to their molar equivalent. It was assumed that 6 moles of O_2 is consumed and 6 moles of CO_2 is produced for each mole of CHO (180 g) oxidised and that the molecular mass of human triacylglycerol is 855.3 $g \cdot mol^{-1}$. The molar rates of triacylglycerol oxidation were multiplied by 3 because each molecule contains 3 moles fatty acid.

6.3.8 Blood sampling and analyses

Blood samples (6-10 mL) were collected into vacutainers containing EDTA and immediately analysed for blood lactate (YSI 2900 STAT Plus, Yellow Springs, OH, USA) and total cholesterol, high density lipoproteins (HDL), low density lipoproteins (LDL) and triglycerides (Cobas b 101, Roche Diagnostics Ltd, Basel, Switzerland). The remaining sample was then centrifuged at 1,500 g for 10 min at 4 °C, and aliquots of plasma were stored at -80 °C for later analysis of FFA (Wako Pure Chemical Industries, Ltd, Osaka, Japan), glycerol (Sigma-Aldrich, Ltd, Australia), insulin (R-Biopharm – Laboratory Diagnostics Pty Ltd, NSW,

Australia), βeta-hydroxybutyrate (βHB) (Sigma-Aldrich, Ltd, Australia) and glucose (Melbourne Pathology, Vic, Australia) concentration.

6.3.9 *Mitochondrial respiration analyses*

Vastus lateralis muscle biopsies were excised and 10-20 mg was immediately placed into 3 mL of ice-cold biopsy preservation solution (BIOPS) [2.77 mM CaK_2 ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 7.23 mM K_2EGTA, 5.77 mM Na_2ATP, 6.56 mM $MgCl_2\cdot6$ H_2O, 20 mM taurine, 15 mM Na_2Phosphocreatine, 20 mM imidazole, 0.5 mM dithiothreitol (DTT), 50 mM MES hydrate; pH 7.1]. Muscle fibers were mechanically separated in ice-cold BIOPS to maximise fiber surface area and transferred into ice-cold BIOPS supplemented with saponin (50 $\mu g\cdot mL^{-1}$) for 30 min with agitation to permeabilise the sarcolemma and allow diffusion of substrates. Fibers were then washed 3 times via agitation in ice-cold MiR05 respiration medium (20 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), 0.5 mM EGTA, 10 mM KH_2PO_4, 3 mM $MgCl_2\cdot6H_2O$, 60 mM lactobionic acid, 20 mM taurine, 110 mM D-sucrose, 1 $g\cdot L^{-1}$ bovine serum albumin (BSA); pH 7.1). Fiber bundles were divided and weighed on a microbalance (1.5-3 mg each) for respirometry analysis in duplicate. All respiration analyses were commenced within 1 h of sampling.

Electron transport system (ETS) and oxidative phosphorylation (OXPHOS) respiration were measured by the Oxygraph O2k high resolution respirometer (Oroboros Instruments, Innsbruck, Austria) via a substrate-uncoupler-inhibitor titration (SUIT) protocol at 37 °C in MIR05 respiration medium with magnetic stirring at 750 rpm. Briefly, after fibers were added and O_2 was injected to the respiration chamber (maintained between 300 and 500 pmol), the sequential addition SUIT protocol commenced with titrations of malate (2 mM final

concentration) and octanoylcarnitine (0.2 mM) to determine leak electron-transferring flavoprotein (ETF) respiration. OXPHOS ETF (ETFp) respiration was assessed by addition of adenosine diphosphate (ADP; 5 mM), complex I (CI) substrate pyruvate (5 mM) and complex II (CII) substrate succinate (10 mM). Cytochrome c (10 μM) was added to confirm mitochondrial membrane integrity, and titrations of carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP; 0.025 μM) were added to determine uncoupled respiratory flux. Complex-specific respiration was inhibited by the addition of rotenone (1 μM) and antimycin A (5 μM) to CI and complex III (CIII), respectively. Finally, complex IV (CIV) capacity was measured during oxidation of N,N,N',N'-Tetramethyl-p-phenylenediamine dihydrochloride (TMPD; 0.5 mM) with ascorbate (2 mM). O_2 flux due to auto-oxidation of these chemicals was determined after inhibition of complex IV (CIV) with sodium azide (15 mM) then subtracted from the raw CIV O_2 flux. Chamber O_2 concentration was maintained between 300 and 450 μmol$^.$L^{-1}. Mass-specific O_2 flux was determined from steady-state flux normalised to tissue wet weight and adjusted for instrumental background and residual O_2 consumption.

6.3.10 *Muscle glycogen concentration*

Muscle glycogen concentration was determined as described previously (Churchley et al., 2007). In brief, ~20 mg of muscle was freeze-dried and powdered, with all visible connective tissue removed under a microscope. Glycogen was then extracted from the freeze-dried sample and glycogen concentration was determined via enzymatic analysis (Passonneau & Lauderdale, 1974).

6.3.11 Citrate synthase activity

Whole skeletal muscle lysates were prepared at a concentration of 2 mg·mL^{-1} and 5 μL of sample was loaded onto a 96-well microtiter plate with 40 μL of 3 mM acetyl CoA, and 25 μL of 1 mM 5,5'-dithiobis [2-nitrobenzoic acid] (DTNB) in 165 μL of 100 mM Tris buffer (pH 8.3). Subsequently, 15 μL of 10 mM oxaloacetic acid was added to each well and immediately analysed using a SpectraMax Paradigm plate reader (Molecular Devices, Sunnyvale, CA). Absorbance was read at 412 nm and was recorded every 15 s for 3 min after 30 s of linear agitation. Maximal activity was recorded with citrate synthase activity reported in mol·h·kg^{-1} protein.

6.3.12 Protein analyses

For generation of whole skeletal muscle lysates, ~40 mg of skeletal muscle was homogenised in buffer containing 50 mM Tris·HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM DTT, 10 μg/mL trypsin inhibitor, 2 μg·mL^{-1} aprotinin, 1 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride. Samples were spun at 16,000 g for 30 min at 4°C and supernatant was collected. After determination of protein concentration via bicinchoninic acid protein assay (Pierce, Rockford, IL), lysates were resuspended in Laemmli sample buffer and 10 μg protein of each sample was loaded into 4–20% Mini-PROTEAN TGX Stain-Free Gels (Bio-Rad Laboratories, California, USA). For OXPHOS antibody cocktail, 8.5 μg protein from unboiled lysates were loaded into 12% polyacrylamide gels. Following electrophoresis, gels were activated according to the manufacturer's instructions (Chemidoc; Bio-Rad Laboratories, Gladesville, Australia) and transferred to polyvinylidine fluoride (PVDF) membranes. After transfer, a Stain-Free image was obtained for protein loading normalisation before rinsing

membranes briefly in distilled water, blocking for 1 h with 5% nonfat milk, washing three times (5 min each wash) with 10 mM Tris·HCl, 100 mM NaCl, and 0.02% Tween 20 solution (TBST) and incubating with primary antibody diluted in TBST (1:1,000) overnight at 4°C on a shaker. Membranes were incubated for 1 h the next day with a secondary antibody diluted in TBST (1:2,000) and proteins were detected via enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK; Pierce Biotechnology) and quantified by densitometry (Chemidoc; Bio-Rad Laboratories). Time points and both diets for each subject were run on the same gel.

Antibodies against fatty acid translocase (FAT/CD36) (no. 14347), Carnitine palmitoyltransferase-1 (CPT1A) (no. 12252), AMP-activated protein kinase (AMPKα) (no. 2532), phospho-AMPK^{Thr172} (no. 2531), Acetyl CoA Carboxylase (ACC) (no.3662), phospho-ACC^{Ser79} (no.3661), mammalian target of rapamycin (mTOR) (no. 2972), phospho-mTOR^{Ser2448} (no. 2971), S6 Ribosomal Protein RPS6 (no. 2217), phospho-RPS6$^{Ser235/236}$ (no. 2211), Citrate Synthase (no. 14309), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (no. 2118) were purchased from Cell Signaling Technology (Danvers, MA) and total OXPHOS (no. 110411) purchased from Abcam (Cambridge, UK). Volume density of each target band was normalised to total protein loaded into each lane using Stain-Free technology (Bio-Rad Laboratories), excluding OXPHOS cocktail which was normalised to GAPDH imaged from the same membrane following the addition of stripping buffer (Thermo Fisher Scientific) to the OXPHOS membrane and re-probing for GAPDH. Following protein loading normalisation, each phosphoprotein was then normalised to its respective total protein.

6.3.13 Statistics

Statistical analysis was undertaken using SPSS (Version 20 for Windows, SPSS Inc, Chicago, IL). Data from the two experimental conditions were analysed using a linear mixed model (treatment × time) and subsequent *post hoc* comparisons were completed within the linear mixed model based on least significant difference. Separate analysis was completed to compare day five of high-CHO diet to 100SS (fed) and day one of HFAT or HPRO to five days post-diet (fasted). Normality was visually assessed using the linear model residuals. Differences in TT performance between trials were compared using a Student's paired t-test. Statistical significance was considered at $P < 0.05$. All data are represented as mean ± SD.

6.4 Results

All participants complied with the prescribed dietary (Table 1) and training intervention for both conditions. No difference was reported across the 5-day intervention periods for distance covered or RPE during training for either diet (HFAT, 222 ± 23 km, 13 ± 0.5; HPRO, 196 ± 29 km, 13 ± 0.7 respectively).

6.4.1   Muscle glycogen concentrations

There was a significant main effect of time for muscle glycogen concentration ($P <$ 0.001) (Figure 6.2). Muscle glycogen was reduced in both HFAT and HPRO conditions pre- to post-diet ($P < 0.001$). Following one day of high-CHO diet, muscle glycogen increased by ~45% in both HFAT and HPRO conditions ($P < 0.001$) but was not restored back to pre-diet values in HFAT ($P < 0.001$). Following 100SS, muscle glycogen was reduced in both HFAT and HPRO conditions (526 ± 86 to 411 ± 62 mmol·kg^{-1} dry mass (DM), P=0.033; 637 ± 87 to 420 ± 92 mmol·kg^{-1} DM, $P < 0.001$, respectively); however, no difference in the percentage change from pre- to post-exercise was measured between conditions.

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6.4.2 Rates of substrate oxidation

There was a significant interaction effect for RER and rates of CHO and fat oxidation (all P = 0.001) (Figure 6.3A, B, C) after five days of either HFAT or HPRO diet. RER was reduced pre- to post-diet for both HFAT and HPRO (0.90 ± 0.02 to 0.79 ± 0.02; 0.90 ± 0.03 to 0.86 ± 0.02, P ≤ 0.001 respectively) and was lower post-diet in HFAT compared to HPRO (P < 0.001). Rates of fat oxidation increased after five days of HFAT and HPRO and were greater in HFAT compared to HPRO post-diet (55 ± 7 vs. 36 ± 6 μmol·kg·min^{-1}, P < 0.001). Concomitantly, rates of CHO oxidation were reduced pre- to post-diet in both conditions and were lower in HFAT than HPRO post-diet (106 ± 20 μmol·kg·min^{-1} vs. 169 ± 17 μmol·kg·min^{-1}, P < 0.001). Following one day of high-CHO diet, RER values returned to baseline in the HPRO trial during the first 20 min of 100SS, but remained lower than baseline in the HFAT trial (HFAT 0.93 ± 0.02 to 0.90 ± 0.03, P = 0.002). RER was lower in HFAT compared to HPRO during the first 40 min of 100SS (P < 0.04). Following one day of high-CHO diet, rates of CHO oxidation were lower than baseline during 100SS following HFAT (P < 0.001) and were significantly lower during 100SS in HFAT compared to HPRO (213 ± 35 vs. 241 ± 31 μmol·kg·min^{-1}, P = 0.025, respectively). Rates of CHO oxidation declined throughout 100SS in HPRO (from 241 ± 31 to 215 ± 26 μmol·kg·min^{-1}, P < 0.001), but remained stable in HFAT (~208 μmol·kg·min^{-1}). Despite one day of high-CHO diet, rates of fat oxidation remained elevated above baseline in the HFAT trial during the first 20 min of 100SS (P = 0.002) but returned to baseline in HPRO. During the first 20 min of 100SS, rates of fat oxidation were significantly higher in HFAT than HPRO (0.53 ± 0.11 vs. 0.38 ± 0.18 μmol·kg·min-1, P = 0.010) and remained higher than HPRO after 40 and 80 min of exercise. Rates of fat oxidation increased during 100SS in both HFAT and HPRO (P < 0.05).
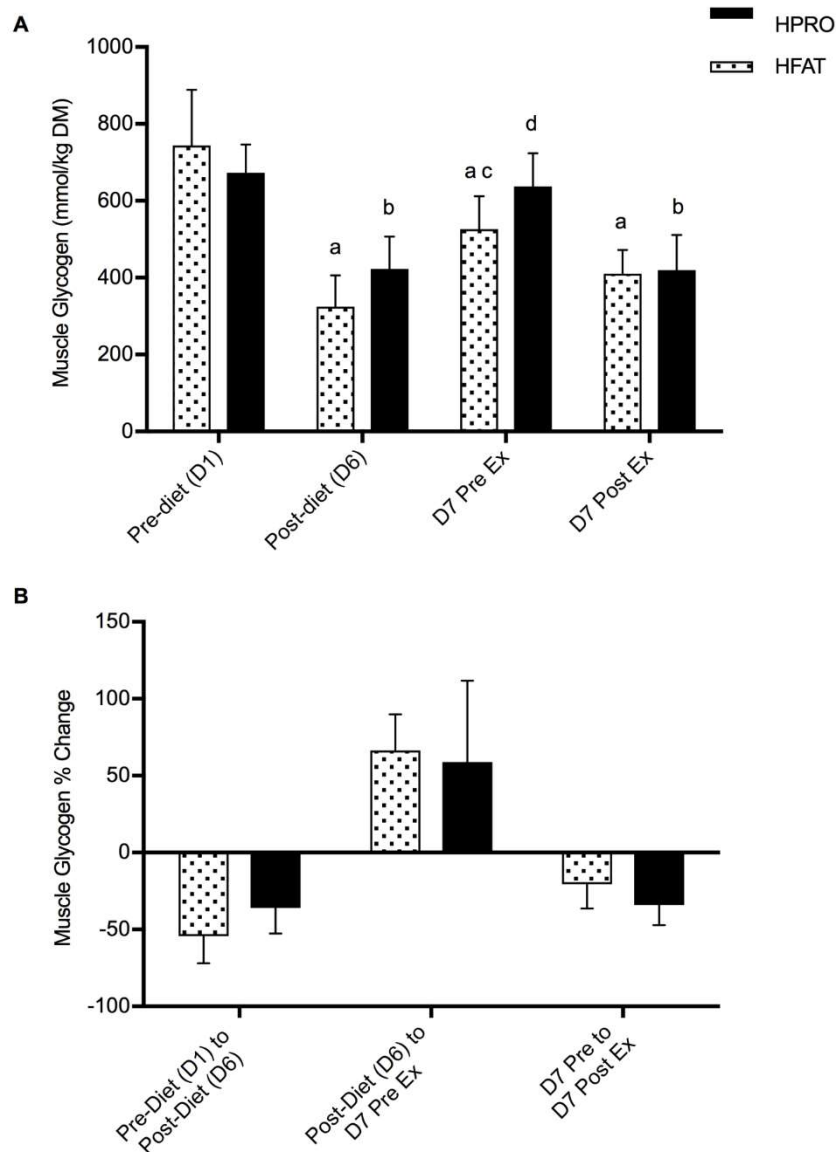
Figure 6.2. Resting muscle glycogen levels following five days high-CHO (Pre-diet D1), five days high-fat or high-protein (Post-diet D6), one day of a high-CHO diet (D7 Pre Ex) and following 100 min SS cycling at 63% PPO (D7 Post Ex) (A) and muscle glycogen percent change from Pre-diet (D1) to Post-diet (D6), from Post-diet (D6) to D7 Pre Ex and from D7 Pre Ex to D7 Post Ex (B) . Values are mean ± SD for n=6. a HFAT different to Pre-diet (D1) within condition; b HPRO different to Pre-diet (D1) within condition; c HFAT different to D7 Pre Ex and D7 Post Ex within condition; d HPRO different to D7 Pre Ex and D7 Post Ex within condition.
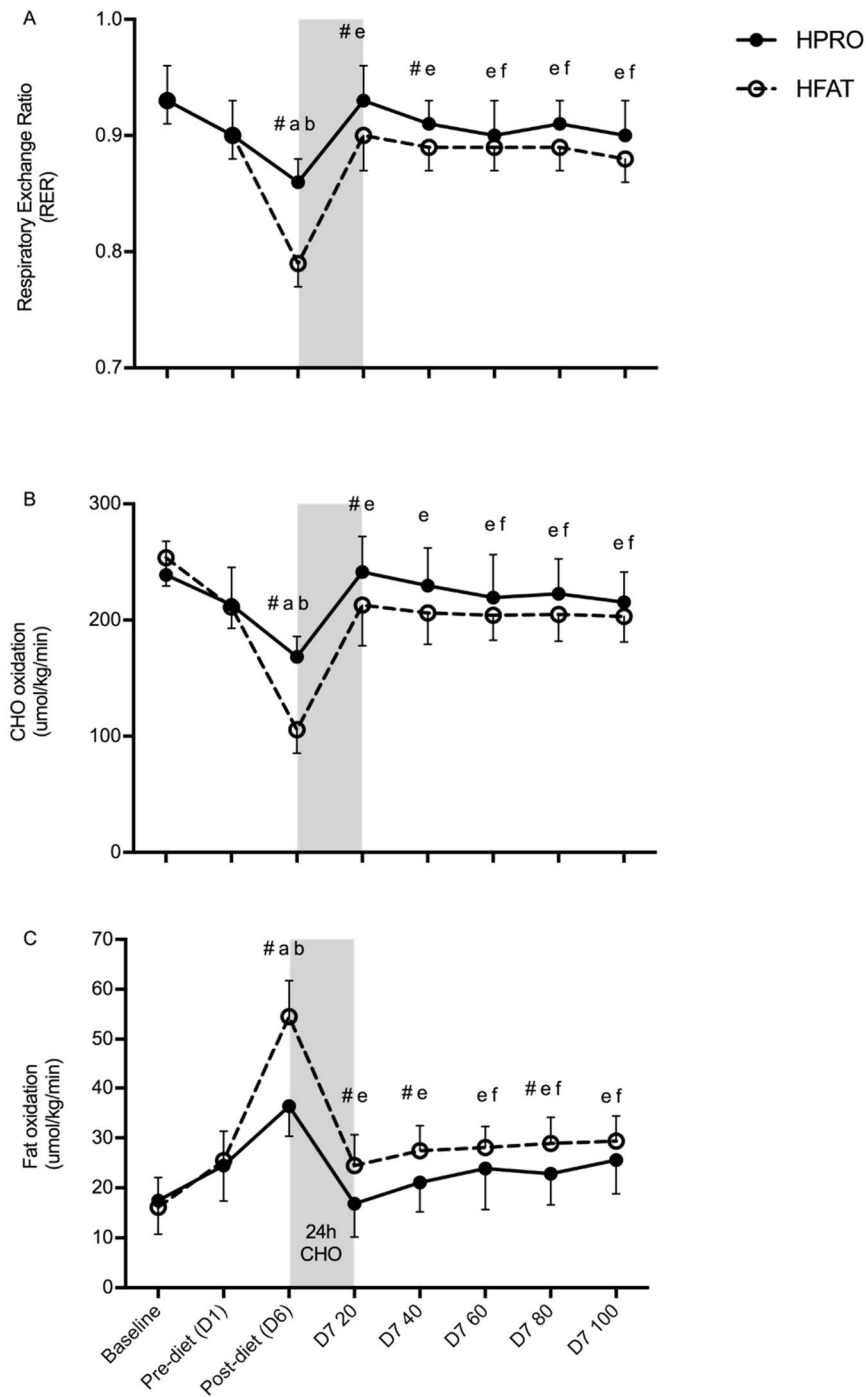
Figure 6.3. RER (A) and rates of CHO (B) and fat oxidation (C) following five days high CHO (Baseline and Pre-diet D1), five days high-fat or high-protein (Post-diet D6) during 20 min cycling and following one day of a high-CHO diet (D720-100) during 100 min SS cycling at 63% PPO. Values are mean ± SD. # HFAT different to HPRO at time point; e HFAT different to Baseline within condition; f HPRO different to Baseline within condition; a HFAT different to Pre-diet (D1); b HPRO different to Pre-diet (D1) within condition.

6.4.3 Blood metabolites pre- and post-diet

There was a main effect of time for FFA concentration (P < 0.001) pre- to post-diet. FFA concentration was greater following exercise post-diet compared to pre-diet in both HFAT (0.31 mM to 0.58 mM, P < 0.001) and HPRO (0.33 to 0.56 mM, P < 0.001). There was a significant main effect of time (P = 0.013) and condition (P = 0.048) for LDL cholesterol. LDL cholesterol increased pre- to post-diet in HFAT (2.44 ± 0.63 to 2.93 ± 0.75 mM) and was higher than HPRO post-diet (2.93 ± 0.75 vs 2.55 ± 0.71, P = 0.025). There was a significant interaction for HDL cholesterol and triglycerides (P = 0.010, 0.042, respectively) between HFAT and HPRO. HDL cholesterol increased (1.01 ± 0.20 to 1.30 ± 0.23 mM, P < 0.001) pre-to post-diet to be greater than HPRO (1.08 ± 0.20 mM), while triglycerides decreased (1.26 ± 0.46 to 0.69 ± 0.36 mM, P = 0.001) to be lower than HPRO (1.06 ± 0.45 mM). No difference in total cholesterol was measured between conditions from pre- to post-diet.

6.4.4 Blood metabolites during the performance ride

There was a significant interaction for plasma glycerol concentration between HFAT and HPRO (P = 0.035; Figure 6.4A). Glycerol concentration increased significantly from rest after 60 min of exercise in HFAT and remained elevated until after the TT. Glycerol concentrations were significantly higher in HFAT than HPRO after 40 min of 100SS. There was a significant effect of time for plasma FFA (P < 0.001), although no differences were observed between diets (Figure 6.4B). FFA concentrations decreased 2 h following CHO breakfast in both conditions and were elevated from resting values after 60 min of 100SS until completion of the TT. Plasma βHB concentrations increased following CHO breakfast and remained stable during 100SS in both conditions until after the TT (Figure 6.4C). There was a main effect of time for blood lactate, blood glucose and plasma insulin concentrations (P <

0.001) during 100SS, but no differences between diets (Figure 6.4D-F). Blood glucose concentration decreased following CHO breakfast in both diets but following 40 min of exercise, glucose concentrations had increased back to resting values. Plasma insulin concentrations increased in both conditions after breakfast and remained elevated 2 h after ingestion. After onset of exercise, insulin concentrations were reduced in both conditions and were similar to pre-breakfast values throughout 100SS. Following 100SS, participants ingested a CHO drink which increased insulin concentrations in both conditions, but this increase was abolished following the onset of the TT. Blood lactate concentrations remained stable throughout 100SS in both conditions and were higher post TT compared to rest in HFAT (3.1 $\pm$ 1.1 mM) and HPRO (3.1 $\pm$ 1.0 mM).

6.4.5 TT Performance

There was no difference in TT performance between diet conditions (30:59 $\pm$ 2:55 vs. 30:10 $\pm$ 2:70 min:sec for HFAT and HPRO, respectively). Mean PO during the TT were 299 $\pm$ 34 W and 304 $\pm$ 35 W (P = 0.41) and HR averaged 168 $\pm$ 9 bpm and 166 $\pm$ 7 b.min^{-1} in HFAT and HPRO, respectively. A significant reduction in BM was observed pre- to post-exercise (P < 0.04) for both HFAT (-0.86 $\pm$ 0.79 kg) and HPRO (-0.61 $\pm$ 0.83 kg), although there were no differences between conditions. No difference in RPE was reported between conditions during 100SS although RPE increased throughout the exercise in both HFAT and HPRO (from 11 $\pm$ 1 to 14 $\pm$ 1, P < 0.001).
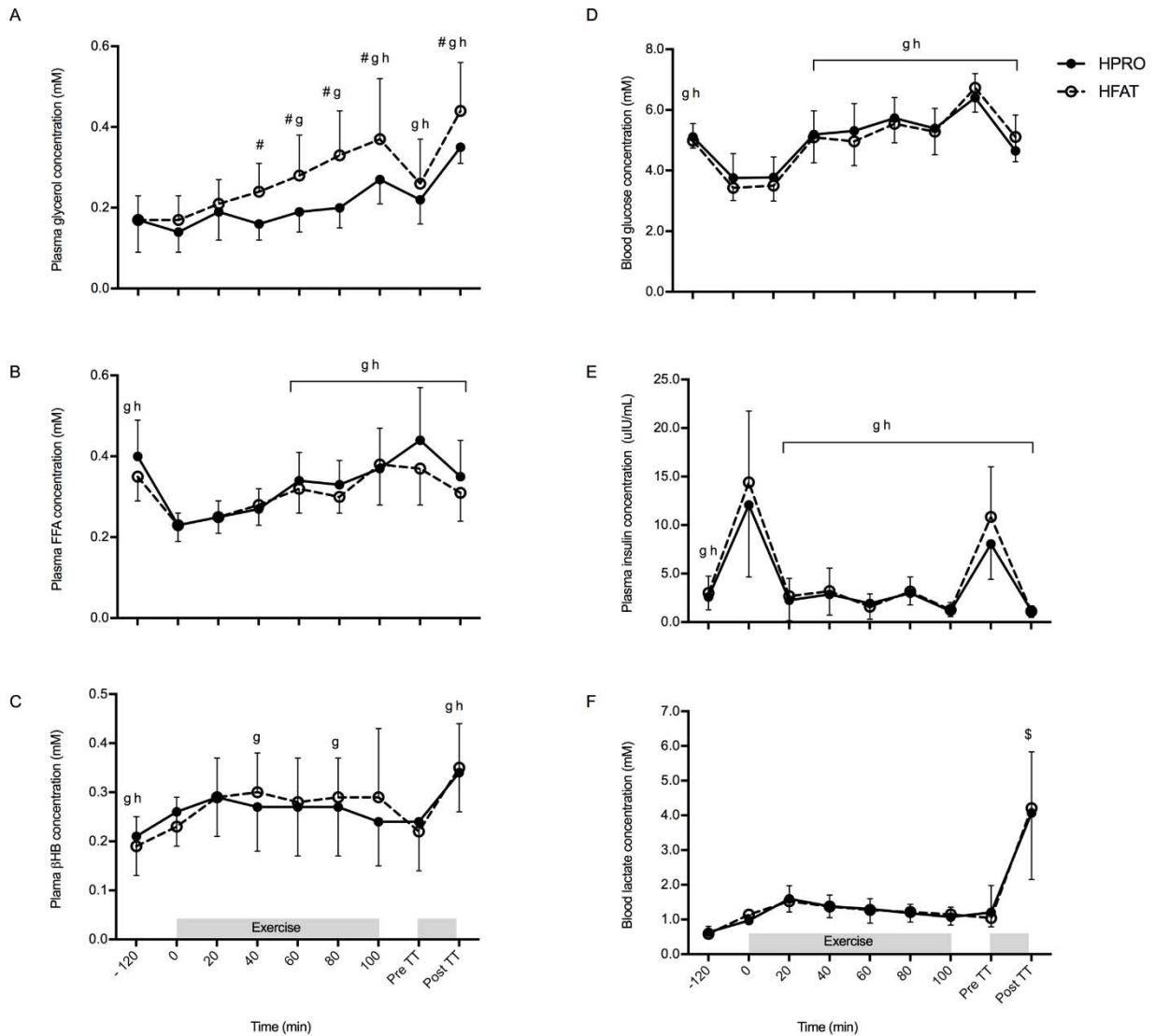
Figure 6.4. Effect of five days high-fat diet or high-protein and one day of a high-CHO diet on plasma glycerol (A), FFA (B), βHB (C), blood glucose (D), plasma insulin (E) and blood lactate concentration (F) before and during 100 min cycling at 63% PPO. Values are mean ± SD. # HFAT different to HPRO at time point; g HFAT different to T=0 within condition; h HPRO different to T=0 within condition; $ different to all time points within each condition.

6.4.6 *Skeletal muscle mitochondrial respiration*

Based on differences in whole-body substrate oxidation rates pre- to post-diet and during prolonged exercise between HFAT and HPRO (Figure 6.3), skeletal muscle mitochondrial substrate utilisation was measured to test if this was contributing to this outcome using a sequential addition SUIT protocol. Absolute O2k respiration measures taken from permeabilised skeletal muscle fiber bundles analysed in duplicate from each participant at each time point and under each diet are reported in Table 6.2. To portray the effects of diet and exercise on mitochondrial respiration, percentage change data are represented in Figure 6.5. Although HFAT and HPRO absolute O2k values were not significantly different following either diet (Table 2), there was a significant interaction in percentage change of CI + ETFp respiration following HFAT and HPRO (P = 0.042; Figure 6.5A). The diet-induced reduction in CI + ETFp respiration following the addition of octanoylcarnitine and pyruvate was significantly greater following HFAT compared to HPRO. Despite no differences in absolute O2k values, percentage change of ETS uncoupled respiration (Figure 6.5A; ETS CI + CII + ETF; ETS CII) was significantly reduced following HFAT but not HPRO. Absolute and percentage change ETS CII uncoupled respiration remained unchanged following one day of high-CHO diet in HPRO but percentage change was significantly increased in HFAT compared to HPRO (P = 0.032, Figure 6.5B). ETFp respiration was significantly reduced following 100SS in HFAT but not HPRO (Figure 6.5C). Percentage change of post-exercise CI + ETFp, CI + CII + ETFp, ETS CI + CII + ETF, and ETS CII respiration was significantly reduced in both HFAT and HPRO (Figure 6.5C) despite no differences in absolute O2k values. The reduction in percentage change of CI + ETFp and CI + CII + ETFp respiration was greater in HFAT than

HPRO following 100SS (P = 0.024, 0.019, respectively). There were no significant differences in skeletal muscle CS activity across time or between diets (~ 20 mol·h·kg^{-1}) (Figure 6.5D).

Table 6.2. Effects of five days high-fat or high-protein diet, one day of a high-CHO diet and 100 min SS cycling at 63% PPO on mitochondrial respiration. Respiratory states are supported by single or convergent electron input via complex I (CI), complex II (CII) and/or electron transfer flavoprotein (ETF) under non-phosphorylating (Leak) conditions, state-3 oxidative phosphorylation in the presence of ADP (indicated by "p"), or with an uncoupler (FCCP) to assess maximal electron transport system (ETS) activity. Values are mean ± SD. a HFAT different to D7 Post Ex; b HPRO different to D7 Post Ex within condition; c HFAT different to D6.

	O2k respiration measure; O2 flux per mass (pmol/(s*mg)); mean ± SD)						
	ETF Leak	ETFp	CI + ETFp	CI + CII + ETFp	cyt-c test	ETS CI + CII + ETF	ETS CII
Substrate/Uncoupler/Inhibitor	malate, octanoylcarnitine	ADP	pyruvate	succinate	cytochrome c	FCCP	Rotenone
HFAT Pre-Diet (D1)	20.2 ± 5.9	43.8 ± 5.8	62.5 ± 11.2 [a]	116.8 ± 22.6 [a c]	136.6 ± 29.5 [a c]	144.4 ± 32.2 [a c]	92.5 ± 19.0 [a c]
HPRO Pre-Diet (D1)	17.5 ± 4.1	47.7 ± 5.6	66.1 ± 15.2	125.0 ± 31.2 [b]	147.1 ± 37.0 [b]	154.9 ± 37.3 [b]	100.6 ± 30.3 [b]
HFAT Post-Diet (D6)	21.0 ± 4.9	42.8 ± 7.0	55.7 ± 13.5	103.5 ± 27.2 [a]	119.1 ± 39.2 [a]	124.0 ± 38.4	77.2 ± 30.5 [a]
HPRO Post-Diet (D6)	19.1 ± 3.8	50.7 ± 4.5	68.4 ± 15.4 [b]	122.0 ± 26.9 [b]	140.3 ± 34.4 [b]	148.2 ± 37.2 [b]	91.3 ± 21.5 [b]
HFAT D7 Pre Ex	24.6 ± 5.7	52.4 ± 7.2	64.3 ± 13.9 [a]	120.1 ± 29.0 [a]	141.1 ± 39.7 [a]	145.9 ± 38.8 [a]	92.9 ± 27.3 [a]
HPRO D7 Pre Ex	18.7 ± 5.9	49.8 ± 12.8	64.8 ± 20.3	120.0 ± 36.9 [b]	142.9 ± 47.3 [b]	151.9 ± 51.0 [b]	97.3 ± 33.6 [b]
HFAT D7 Post Ex	24.6 ± 4.9	44.7 ± 9.8	47.6 ± 13.8	84.0 ± 23.8	99.0 ± 26.7	108.1 ± 31.0	60.8 ± 20.5
HPRO D7 Post Ex	21.7 ± 5.9	47.9 ± 11.3	56.8 ± 18.3	101.1 ± 30.2	105.4 ± 35.4	115.0 ± 37.4	65.4 ± 27.0
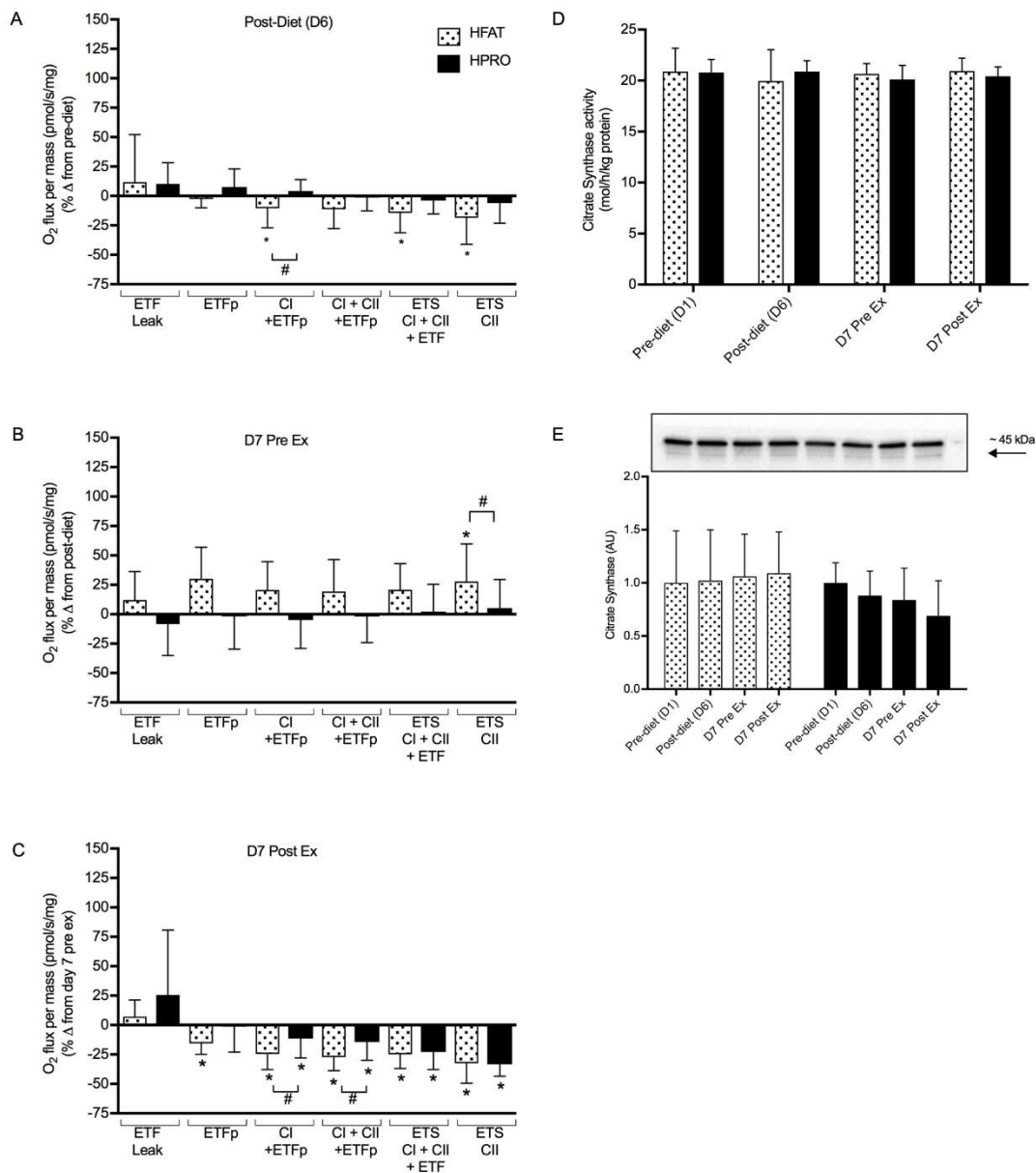
Figure 6.5. Effect of five days high-fat or high-protein diet (A), one day of a high-CHO diet (B), 100 min SS cycling at 63% PPO (C) on mitochondrial respiration using a sequential addition protocol. Respiratory states are supported by single or convergent electron input via complex I (CI), complex II (CII) and/or electron transfer flavoprotein (ETF) under non-phosphorylating (Leak) conditions, state-3 oxidative phosphorylation in the presence of ADP (indicated by "p"), or with an uncoupler (FCCP) to assess maximal electron transport system (ETS) activity. Values are mean ± SD as a fold change from respective day indicated in each figure panel. Differences in respiratory fluxes were not due to altered overall mitochondrial content as indicated by citrate synthase activity (D) or protein content (E). * O_2 flux per mass different from Pre-Diet D1 (A); Post-Diet D6 (B); Pre-Ex D7 (C) of the respective high-fat or high-protein diet. # HFAT different to HPRO condition at time point.

6.4.7 *Immunoblot analyses*

Total protein contents of citrate synthase (Figure 6.5E) and OXPHOS complexes I-V (Figure 6.6A-F) were not different between HFAT and HPRO at any time point during intervention. OXPHOS complex III showed a trend towards a main effect for time ($P = 0.073$) with a decrease from pre- to post HFAT diet. There was a significant interaction for FAT/CD36 protein content ($P < 0.001$) from pre-diet to after one day of high-CHO diet (Figure 6.7A). FAT/CD36 protein content was higher pre- and post-diet and pre- and post-100SS in HFAT compared to HPRO. There were no differences in total CPT1a from pre- to post high-CHO diet in either HFAT or HPRO (Figure 6.7B). No main effects were found for AMPK Thr172 phosphorylation levels relative to total AMPK, although a trend towards a main effect of time was observed ($P = 0.06$) with an increase following HFAT (Figure 6.7C). There was an effect of time for ACC Ser79 phosphorylation relative to total ACC ($P = 0.015$). ACC Ser79 relative to total ACC was greater in HFAT following 100SS compared to post HFAT (Figure 6.7D). There were no differences in mTOR Ser2448 phosphorylation relative to total mTOR pre-diet compared to after one day of high-CHO (Figure 6.7E). There was a significant effect of time for RPS6 Ser235/236 phosphorylation relative to total RPS6 ($P < 0.05$). RPS6 Ser235/236 phosphorylation increased following 100SS in HPRO compared to pre- and post-diet (Figure 6.7F). RPS6 Ser235/236 phosphorylation was also higher post-exercise in HPRO compared to HFAT ($P = 0.034$).
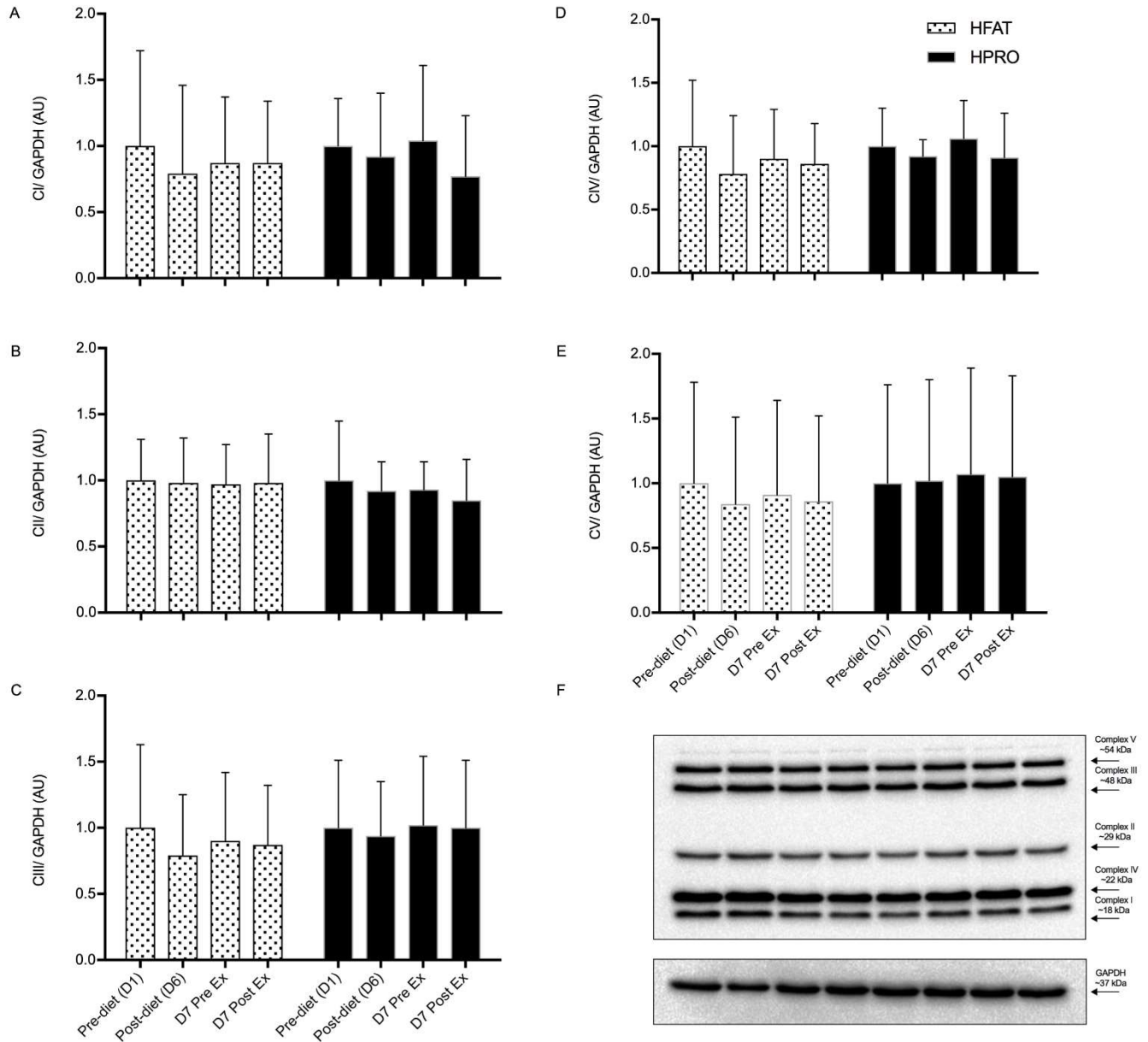
Figure 6.6. Skeletal muscle protein content (A-E) and representative images (F) of five OXPHOS complexes following five days high-CHO diet, five days high-fat or high-protein diet, one day of a high-CHO diet and following 100 min SS cycling at 63% PPO . Values are mean ±SD as a fold change relative to resting pre-diet D1 values.
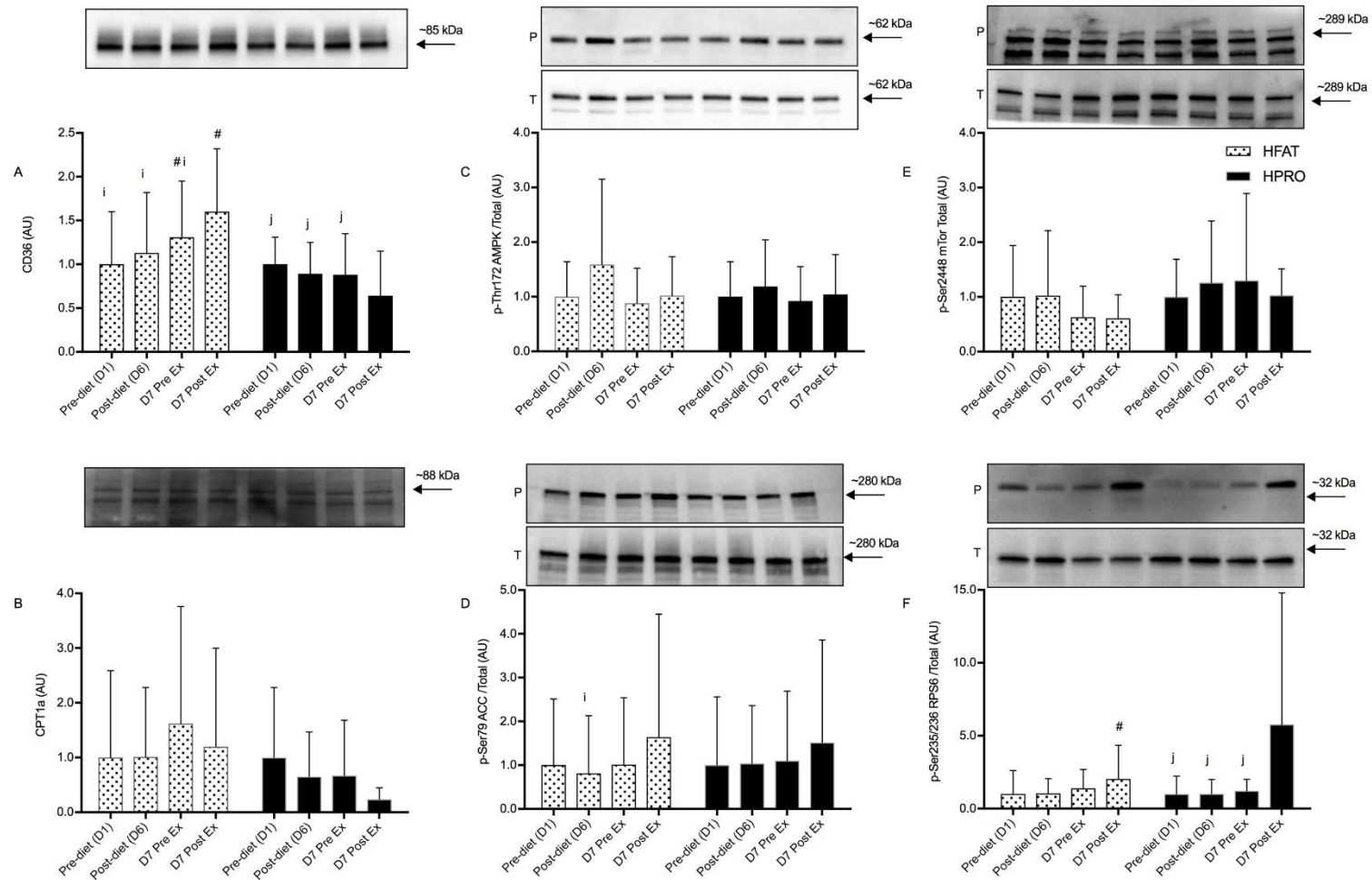
Figure 6.7. Skeletal muscle protein content and representative blots of fat (CD36, CPT1a) (A & B), fat/CHO (p- Thr172 AMPK/Total, p-Ser79 ACC/Total) (C & D) and protein regulatory signaling pathways (p-Ser2448 mTOR/Total, p-Ser235/236 RPS6/Total) (E & F) following five days high-CHO, five days high-fat or high-protein diet, one day of a high-CHO diet and following 100 min SS cycling. Values are mean ± SD as a fold change relative to resting pre-diet D1 values. # HFAT different to HPRO at time point; i HFAT different to D7 Post Ex within condition, j HPRO different to D7 Post Ex within condition.

6.5 Discussion

This is the first study to manipulate dietary fat and protein content while simultaneously 'clamping' dietary CHO intake during a short-term period of intense exercise training in well-trained humans. Such an experimental design is essential in an effort to pinpoint potential mechanisms underlying the high rates of fat oxidation reported following short-term adaptation to fat-rich diets, which persist even after one day of glycogen restoration with high-CHO intake (Burke et al., 2000; Burke et al., 2002) and/or high exogenous CHO availability (Burke et al., 2002; Carey et al., 2001; Stellingwerff et al., 2006). The results of the present study provide novel insights into the mechanisms governing patterns of substrate oxidation in response to diet-exercise interactions. It was reported that compared to an isoenergetic high-protein diet, five days' adaptation to a high-fat diet results in greater whole-body rates of fat oxidation during submaximal cycling and impairments in mitochondrial respiration.

A series of independent studies over the past two decades (Burke et al., 2000; Burke et al., 2002; Carey et al., 2001; Havemann et al., 2006; Stellingwerff et al., 2006) have compared high-fat versus high-CHO diets and shown that short-term (< 7 days) high-fat diets result in peak rates of whole-body fat oxidation of ~ 1 $g \cdot min^{-1}$ (~ 50 $umol \cdot kg \cdot min^{-1}$), values that are typically two-fold greater than after isoenergetic high-CHO diets (Burke et al., 2000; Burke et al., 2002). The rates of fat oxidation in the present investigation (1.2 $g \cdot min^{-1}$) after an identical period of a fat-rich diet were similar to those reported previously. However, the first novel finding from the present study was that rates of fat oxidation were 33% greater than after five days of a low-CHO, HPRO diet (0.8 $g \cdot min^{-1}$). An increase in post-exercise plasma FFA concentration from pre-diet interventions in HFAT and HPRO likely contributed to the increased rates of fat oxidation after both conditions. However, the higher rates of fat oxidation

in HFAT compared to HPRO are likely associated with altered rates of whole body lipolysis and subsequent storage of triglycerides (Cameron-Smith et al., 2003; Yeo et al., 2008; Zderic et al., 2004). Previous work has shown higher rates of whole-body lipolysis, determined by elevated glycerol concentration, and this increase was associated with elevated intramuscular triglyceride (IMTG) concentration following fat-adaptation (Helge et al., 2002; Zderic et al., 2004). Limited muscle biopsy sample did not permit IMTG measurements in the current study. It is also known that low-CHO availability reduces circulating insulin concentrations which could increase rates of whole-body fat oxidation. Although CHO intake was identical in both dietary conditions, it is likely that a proportion of protein in HPRO was converted to glucose via gluconeogenesis (Wolfe et al., 1977), which may explain slightly higher muscle glycogen concentrations post-diet in HPRO compared to HFAT. Higher availability of muscle glycogen likely contributes to lower rates of whole-body fat oxidation in HPRO compared to HFAT. Accordingly, the higher rates of fat oxidation measured after HFAT are likely driven by higher fat rather than higher CHO availability.

Despite CHO intake being 'clamped' in both dietary conditions, rates of CHO oxidation were lower following five days HFAT compared to HPRO. Rates of CHO oxidation were reduced by 50% (3 $g \cdot min^{-1}$ to 1.5 $g \cdot min^{-1}$) following HFAT compared to a 25% decline (2.3 $g \cdot min^{-1}$) following HPRO. The greater oxidation of CHO-based fuels may be explained by the slightly higher muscle glycogen concentration post-diet in HPRO compared to HFAT, which has previously been shown to increase reliance on CHO-based fuels during subsequent exercise (Bergstrom et al., 1967). Without a protein tracer in the current study, it was not possible to detect the proportion of protein that is being converted to glucose or calculate rates of protein oxidation. Following a 6-day high-fat diet (63% EI from fat), Peters et al. (1998) observed a

decrease in the active form of the rate limiting enzyme in CHO metabolism, pyruvate dehydrogenase (PDH), and consequently a reduction in rates of CHO oxidation during exercise, which was not observed following a moderate CHO diet (52% EI CHO). Therefore, a decrease in PDH activity may contribute towards the observed reduction in whole-body rates of CHO oxidation in the present study (Stellingwerff et al., 2006); however, limitations in muscle biopsy sample did not permit assessment of PDH activity.

To further determine potential mechanisms for reduced CHO oxidation, skeletal muscle mitochondrial respiration was assessed to measure dietary effects on substrate flux and utilisation. Respiration supported by octanoylcarnitine and pyruvate (CI + ETFp) was significantly reduced after five days of HFAT, but not HPRO, when CIII and/or CIV are operating at or near maximal activity. In our interpretations of mitochondrial respiration data obtained using the Oroboros O2k SUIT protocol, it is important to note that supra-physiological mitochondrial substrate concentrations and a sequential addition protocol are used. Therefore, interpretations of substrate-specific effects on respiration must be made with caution because this protocol does not allow us to pinpoint whether the addition of a particular substrate alone or any previously added substrate in the protocol are responsible for the effect. Nonetheless, it was surprising that FFA (i.e. octanoylcarnitine)-driven mitochondrial respiration (ETF leak) was not subsequently increased with HFAT. Decreased respiration observed following the addition of octanoylcarnitine and pyruvate is in line with previous studies reporting high-fat diets reduce the amount of PDH (in its active form [PDHa]) and PDHa activity at rest but not after a moderate CHO diet (Peters et al., 1998). Alterations in PDH activity have further been identified as a mechanism underlying regulation of metabolic flexibility in isolated rodent skeletal muscle mitochondria in response to altered substrate availability induced by high-fat

feeding (Jorgensen et al., 2017). In addition, the reduction in respiration after five days of HFAT persisted after uncoupling (ETS CI + CII + ETF and ETS CII), suggesting that the functional reductions in respiration occurred either at the level of CI/CII or downstream at CIII/CIV but not at CV (ATP-synthase). In line with the observed reductions in uncoupled respiration, Skovbro et al. (2011) observed that ETFp and uncoupled respiration were decreased following a longer high-fat feeding period (i.e. 16 days; 55-60% fat) compared to a moderate CHO diet (i.e. 55-60% CHO) (Skovbro et al., 2011). The mitochondrial effects of HFAT in the present study may have been more pronounced following a longer dietary intervention period. Additionally, the type of the dietary fat intake has previously shown to affect mitochondrial function and morphology (Lionetti et al., 2014). Lionetti et al. (2014) has shown that high saturated fat intake was associated with greater mitochondrial dysfunction compared to unsaturated fat in rodents. The current study provided a 65% fat diet, which was made up of 55% saturated fat, and whether the reductions in mitochondrial respiration would be observed with a different dietary fat composition requires further investigation. As no changes in mitochondrial respiration were observed following five days of HPRO, this suggests that the primary driver of these skeletal muscle adaptations is high dietary fat availability. Based on the evidence in the present study, the biochemical explanation of why HFAT results in increased whole-body fat oxidation despite reduced skeletal muscle mitochondrial respiration is inconclusive and warrants future investigation of mitochondria in other tissues. Given that potential HFAT-induced changes in the delivery and transport of fatty acids across sarcolemma are removed in the ex vivo analysis of mitochondrial respiration, these additional variables may also contribute to the observed increases in fat oxidation at the whole-body level.

Although changes were observed in respiration following the addition of octanoylcarnitine and pyruvate and uncoupled respiration with HFAT, no difference was detected in skeletal muscle protein content of the five mitochondrial OXPHOS complexes after either dietary condition. Additionally, neither citrate synthase protein content nor maximal activity was changed. Given that changes in complex I and citrate synthase activity have strong associations with mitochondrial content (i.e. volume and/or density), this suggests that content is not affected by either short-term HFAT or HPRO availability (Larsen et al., 2012). This is in contrast to findings by Skovbro et al. (2011) where a reduction in complex 1 protein expression was reported and suggested to contribute towards the reduced respiratory glycolytic flux. The difference in findings with the current study may be associated with the longer duration of the dietary intervention. It is speculated that changes in enzyme activities regulating mitochondrial substrate flux also likely contribute towards the reduced respiration observed after short-term adaptation to HFAT.

To determine alternative enzymes and signaling pathways impacted by the HFAT and HPRO, putative transporters with roles in skeletal muscle FA uptake, and two energy sensing metabolic signaling pathways, AMPK and mTOR were measured. There was a 12% increase in FAT/CD36 protein observed following HFAT, suggesting potential increased capacity for sarcolemmal and/or mitochondrial membrane FA uptake, although this increase in FAT/CD36 did not reach statistical significance. No change in mitochondrial CPT1 was observed following five days of HFAT or HPRO. These findings are in agreement with previous work which reported that a high-fat diet together with an intensive training program resulted in significantly greater (i.e. 17% increase) protein abundance of FAT/CD36 without change in gene expression of CPT1 (Cameron-Smith et al., 2003). Low CHO diets together with periods of endurance

training have previously been shown to increase AMPK activation and signaling to its downstream substrate ACC (Steinberg et al., 2006). However, no significant change was reported in AMPK Thr172 phosphorylation relative to total AMPK, or its substrate ACC Ser79 phosphorylation relative to total ACC following the dietary interventions. No differences post-diet were found in mTOR Ser2448 phosphorylation relative to total mTOR and phosphorylation of its substrate RPS6 Ser235/236 relative to total RPS6 in HFAT or HPRO. Muscle samples for western blotting were not freeze dried and dissected for non-muscle tissue which can introduce a small amount of variability in the findings, this must be considered when interpreting findings. However, together these findings suggest that FA transporter abundance and activation of these energy-sensing pathways were unaffected by the two diet interventions, perhaps as a result of the high training status of the cyclists and the ability to cope with the demand of the dietary overload. Further investigation is required to uncover alternative protein signaling pathways associated with changes in substrate metabolism that may underpin the dietary effects on skeletal muscle mitochondrial respiration.

After CHO restoration strategies (e.g. 1 day of high-CHO diet, a pre-exercise CHO-rich breakfast and CHO intake during exercise) muscle glycogen increased in both HFAT and HPRO, but did not reach pre-intervention values in HFAT. This may be a result of the brief (24 h) CHO restoration period compared to five days of high CHO intake prior to baseline measures. Rates of fat oxidation and CHO oxidation returned towards baseline values during 100 min SS cycling in both HFAT and HPRO and were similar to the results seen in the pre-diet protocol. However, the CHO restoration and exercise feeding protocols involved aggressive strategies to promote high CHO availability from both exogenous and endogenous sources, compared with overnight fasted and water fed conditions on the pre-diet protocol. Therefore, rates of fat

oxidation were higher and CHO oxidation lower than expected during the 100 min steady state protocol on day 7, particularly with HFAT. Indeed, although the present study did not include a direct comparison to a chronic high-CHO diet as in our prior investigations (Burke et al., 2000; Burke et al., 2002; Carey et al., 2001), our results are consistent with previous observations that muscle adaptation during chronic periods of a low-CHO diet, especially in the case of the HFAT, is sufficiently robust to persist despite the restoration of CHO (Burke et al., 2000; Burke et al., 2002; Carey et al., 2001). For example, CHO oxidation rates following HFAT or HPRO and CHO restoration in the current study, were lower than those reported in previous studies following a controlled (chronic high-CHO) diet (200-220 umol·kg·min^{-1} vs. 250-300 umol·kg·min^{-1}) (Burke et al., 2002). Muscle glycogen utilisation in the current study was ~100 mmol·kg DM lower than those previously reported with HFAT intervention [2] and was slightly higher in HPRO compared to HFAT. This difference may be related to higher pre-exercise muscle glycogen in HPRO. Overall, this reduced capacity for CHO oxidation in HFAT, despite the availability of exogenous and endogenous stores was previously associated with persistent downregulation of PDH activity (Stellingwerff et al., 2006).

Whole body rates of fat oxidation after CHO restoration remained slightly higher after HFAT compared to HPRO, but this resulted in only a small difference in the total fat oxidised during 100 min SS cycling (~ 15 g over 100 min). No difference was measured for FFA concentrations between the two dietary conditions, although plasma glycerol concentrations were greater during exercise following HFAT compared to HPRO. The elevated glycerol concentration following HFAT indicates a greater rate of whole-body lipolysis which could be associated with greater IMTG utilisation and/or liberation of FFA into the blood, contributing to the minor variation in rates of whole-body fat oxidation between HFAT and HPRO.

Corresponding to total fat utilisation, there were only minor differences in total CHO oxidised between HFAT and HPRO (~ 30 g) during 100 min of exercise.

Although one day of high CHO availability in the current study failed to fully reverse the differences in whole-body rates of substrate oxidation, it was sufficient to restore the decreased mitochondrial respiration (CI + ETFp) from HFAT, back to pre-diet values. This indicates that there may be an additional underlying mechanism regulating changes in substrate oxidation (i.e. downregulating CHO oxidation) and mitochondrial respiration, for example a downregulation of PDHa. As the current study precluded investigation of a high-CHO trial due to the high number of biopsies that would have been required, it is not possible to speculate whether differences in mitochondrial respiration would have been observed between the three dietary conditions. A greater post-exercise reduction in CI + ETFp and CI + CII + ETFp respiration in HFAT than HPRO was reported and this was not attributable to reductions to citrate synthase protein or activity. These effects of exercise following the HFAT may be attributable to mitochondrial adaptations at the cessation of exercise that impact ETC, including changes in signaling, mitochondrial membrane dynamics and/or buffering of reactive oxygen species. It should also be noted that there could have been damage to myofibers during separation and permeabilisation. This could limit the interpretation of the respiratory values prior to the addition of cytochrome C in the SUIT protocol since it occasionally increased O_2 flux more than 10% above CI+CII+ETFp (Table 2). Despite this, the research team are confident that the data prior to the addition of cytochrome C is still meaningful since the effect was consistent across all trials. To determine the effect of potential shifts in substrate utilisation on exercise performance, previous studies have included a cycling TT after a bout of steady state exercise, following the high-CHO intake. Burke et al. (2000) reported similar TT performance

between high-fat and high-CHO trials and in the present study a difference in TT performance was not detected between HFAT and HPRO after one day of CHO restoration; however, this performance cannot be compared to a high-CHO condition and therefore the shifts in substrate utilisation require further investigation.

In conclusion, the results of the present investigation demonstrate that whole-body rates of fat oxidation increase to a greater extent in trained humans following high dietary fat intake compared to a high-protein diet, with CHO 'clamped' at 20% of energy intake. High dietary fat also reduced mitochondrial respiration supported by octanoylcarnitine and pyruvate as well as uncoupled respiration. These reductions in mitochondrial 'function' may be compensatory, and not solely 'driving' fuel regulation under the conditions of our investigation. Further mechanistic investigation into potential underlying diet-induced differences in mitochondrial membrane dynamics, mitochondrial complex subunits and additional enzymes regulating mitochondrial substrate flux is warranted. The acute but aggressive restoration of endogenous and exogenous CHO availability was unable to completely restore normal rates of substrate oxidation but was able to reverse the fat-induced disruption of mitochondrial respiration. Together these findings demonstrate the impact a high-fat diet has on metabolic flexibility and skeletal muscle mitochondrial respiration in trained cyclists.

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6.5.2 Acknowledgements

6.5.3 Author Conflict

No competing interests declared

7. Chapter 7- General discussion and conclusion

An athlete's nutritional status and use of specific nutritional strategies play a major role in their ability to train for, and compete in, endurance events. Therefore, the main aims of this thesis were to determine the effects of a series of nutritional/training strategies targeting the role of fat as a muscle substrate on adaptation to, and performance of, endurance exercise. Specifically, the studies comprising this thesis examined:

1) The effect of suppressing lipolysis on substrate selection and prolonged, high-intensity running capacity.

2) The effects of exogenous ketone ester ingestion on substrate metabolism and performance.

3) The effects of increased dietary fat availability and a low-CHO intake on the mechanisms underpinning skeletal muscle adaptations.

The findings from these three independent but related studies, represented schematically in Figure 7.1, help inform nutritional guidelines for endurance athletes. Collectively they demonstrate that CHO-based fuels are the predominant substrate for muscular work during prolonged high-intensity exercise.
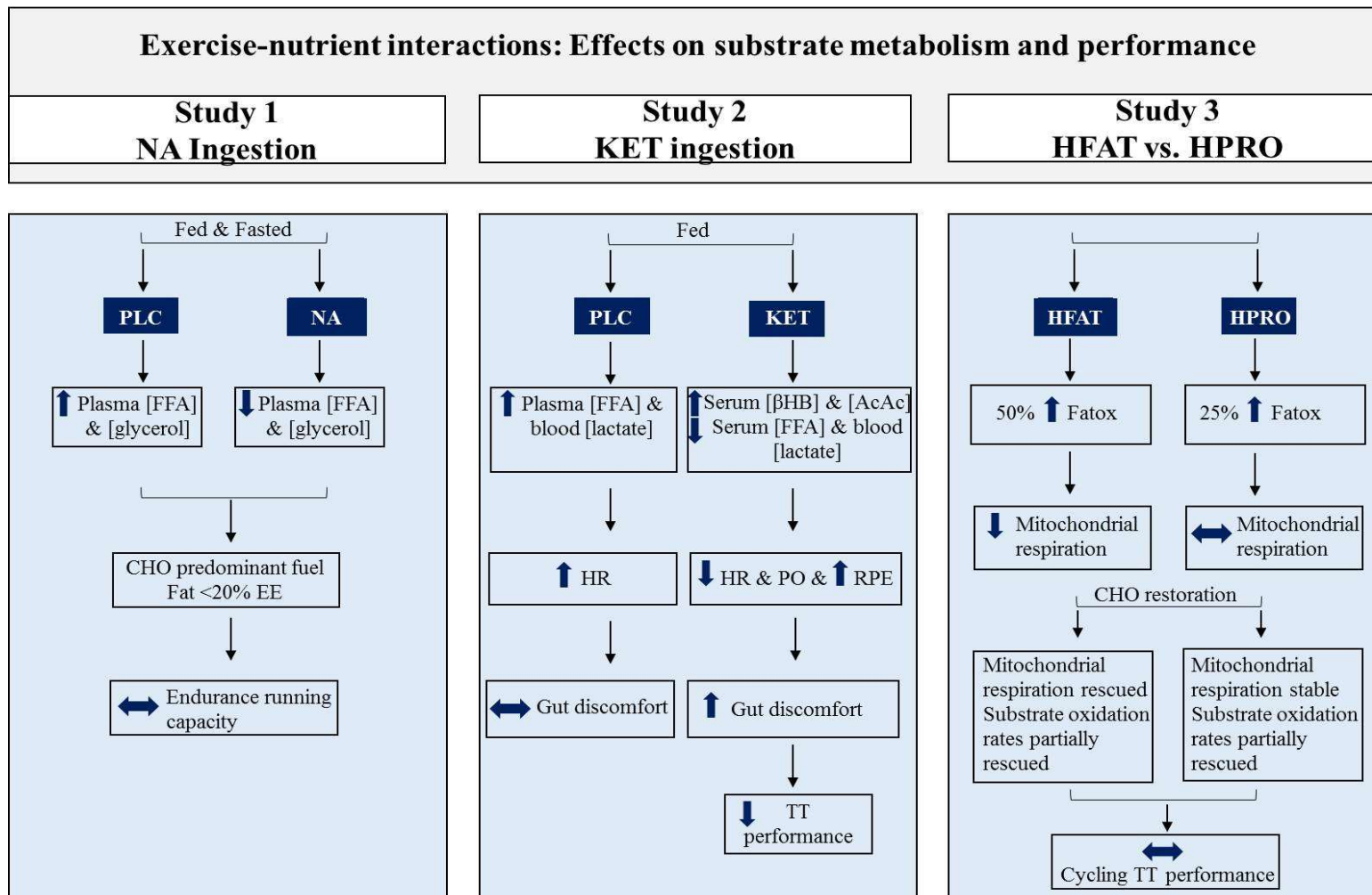
Figure 7.1. Major findings of each study from chapters 4, 5 and 6 of this thesis. NA, Nicotinic acid; PLC, placebo; FFA, free fatty acids; EE, energy expenditure; KET, ketone; βHB, β-hydroxybutyrate; AcAc, Acetoacetate; HR, heart rate; PO, Power output; RPE, rating of perceived exertion; TT, time trial; HFAT, high-fat diet; HPRO, high-protein diet; Fatox, fat oxidation, CHO, carbohydrate.

Few studies to date have investigated fuel utilisation in well-trained athletes at the intensities sustained during training or competition. Due to the dominant role of CHO-based fuels in supporting high-intensity endurance exercise lasting up to ~90 min, it was hypothesised in *study 1* that suppressing lipolysis via NA ingestion would not alter substrate utilisation or have a detrimental effect on prolonged running capacity. In agreement with the research hypothesis, the main finding of *study 1* was that when CHO availability is high, blunting the normal exercise induced rise in FFA did not impair exercise capacity in trained runners. CHO was the predominant fuel source when running at ~80% $\dot{V}O_2$max, providing ~87% of total energy expenditure.

Despite increased CHO availability and reduced circulating FFA following NA administration, there was an obligatory contribution from fat-based fuels (< 20%) for muscular work. It is likely that this fat contribution is derived from IMTGs, however due to the small contribution it is doubtful that this would have been detected via a muscle biopsy sample. It is unknown why fat-based substrates continue to fuel muscular work, even in conditions where CHO availability is high, participants are working at high absolute and relative intensities and when lipolysis is suppressed following NA administration. One possible explanation is that this is an evolutionary process in humans and/or a survival mechanism to preserve the limited stores of endogenous CHO-based substrates under conditions that threaten survival (i.e. starvation, illness, disease). Evolutionary theories suggest that energy availability in human's links back to cycles of feasts and famines throughout the hunter-gatherer period and therefore certain genes have evolved to regulate endogenous fuel storage and utilisation. These have previously been described as the "thrifty genes". Further work is required to determine the underpinning

mechanism/s regulating the utilisation of fat-based substrates, even when the skeletal muscle is primed with CHO-based fuels and endogenous fat availability has purposefully been perturbed.

The second study of this thesis aimed to test the hypothesis that ketone diester ingestion would increase circulating ketones but fail to enhance cycle TT performance lasting 30-40 min. This is because events of this duration and intensity are highly dependent on energy production from CHO-based fuel sources to support the oxidative fuel needs of the working muscles. In support of the hypothesis, results of *study 2* found that ketone ingestion increased circulating ketones. However, when added to nutrition strategies otherwise known to enhance sports performance of this type, the ketone diester ingestion also induced gut discomfort and increased the perception of effort, which lead to impaired TT performance in elite professional cyclists. Regardless of being informed of the detrimental findings from the current study, one of the 11 participants expressed being "possibly" interested in consuming this supplement prior to actual competition. Although athletes would not be encouraged to ingest ketone supplements prior to competition based on the study findings, continued intrigue about these products and the interest of even a small number of elite athletes suggests that further work is necessary. Indeed, there are a number of ketone salts and esters on the market, some of which are widely available while others are yet to be fully commercialised. However, the lack of research on the effects of different ketone supplements on circulating ketones and substrate metabolism make it difficult to make practical recommendations on protocols that could achieve the notional "optimal range" of circulating ketone concentrations (1-3 mM). Indeed, the only available peer-reviewed dose response study concerns a ketone ester which is not commercially available.

Future research should focus on large scale dose response study (ies) involving a range of different ketone esters/salts. To ensure ecological validity, such studies should ensure that

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participants follow nutritional practices that replicate race day strategies (i.e. following a CHO-rich pre-race meal rather than overnight fasted), and also take into account the interaction with the use of other popularly used and evidence-based performance supplements (e.g. caffeine, beetroot juice etc). The effect of ketone supplements on alternative bodily functions that can affect performance should also be explored, as ketone bodies, specifically βHB, have a major effect on the central nervous system, suppressing the sympathetic nerve response. Since the current literature on ketone supplementation has focused on submaximal workloads or short, high-intensity efforts of 30-40 min duration, there is a need for investigations of their effect on exercise protocols that may be limited by CHO availability (i.e. prolonged, high-intensity exercise, > 90 min) and thus may benefit from ketones as an additional oxidative substrate. This proposed series of work on ketones would provide novel information for both athletes and practitioners in determining whether there is any potential benefit of this exogenous oxidative substrate for the working muscle and performance, and if so, how they should be used.

The results from *Studies 1 and 2* demonstrated that although the manipulation of substrate availability can alter fuel selection during exercise, this does not always result in improved sports performance. Indeed, the history of investigation of high-fat, low-CHO diets shows that while they can 'retool' the muscle's fuel preferences in only a few days, causing robust adaptations that persist even following one day of high-CHO feeding, these changes often fail to enhance performance, particularly protocols involving higher intensity workloads. Since the low-CHO, high-fat diet remains topical amongst athletes and researchers, there was merit in further exploring the mechanisms involved in these substrate shifts. The aims of *Study 3* of this thesis were to distinguish between the roles of CHO restriction and high fat availability underpinning the known changes in patterns of substrate oxidation with low-CHO, high-fat

diets. The role of CHO intake was isolated by comparing two CHO-restricted diets (CHO clamped to < 20% of total EI), with the dominant macronutrient being supplied by either high-fat or high-protein food choices.

The major findings of *study 3* were that a high-fat diet increased whole-body rates of fat oxidation during exercise to a greater extent than a high-protein diet in endurance trained cyclists. Fat adaptation also decreased resting skeletal muscle mitochondrial respiration supported by octanoylcarnitine and pyruvate as well as uncoupled respiration, suggesting a reduction in mitochondrial 'function'. One day of CHO restoration and the provision of exogenous CHO sources during exercise were unable to completely restore normal rates of substrate oxidation but appeared to reverse the fat-induced change to mitochondrial respiration. Therefore, although high dietary fat intake increases whole-body rates of fat oxidation, high dietary fat availability is likely to be detrimental to metabolic flexibility (i.e. the ability to transition between substrates) because of the downregulation of key enzymes such as PDHa and changes to skeletal muscle mitochondrial respiration.

This novel finding from *study 3* which identified that fat adaptation reduces muscle mitochondrial respiration supported by octanoylcarnitine and pyruvate as well as uncoupled respiration, opens the door to future studies which should investigate the specific differences in mitochondrial complex subunits and enzymes regulating mitochondrial substrate flux. Although previous work has identified PDH as a key regulator of reduced CHO oxidation following fat adaptation, findings from *study 3* suggest there may be additional factors contributing to the changes in CHO oxidation and mitochondrial respiration. Future work should also explore the effects of different types of dietary fat intake (i.e. saturated vs. unsaturated) on skeletal muscle adaptations to determine if type of fat exacerbates or improves the changes to mitochondrial

function. The composition of fats within different high-fat diets can vary significantly depending on the chosen food sources and this could be contributing to altered mitochondrial function and morphology. For example, previous work in this area has shown that high-saturated fat intake was associated with mitochondrial dysfunction and a shift towards mitochondrial fission compared to high unsaturated fat intake which increased mitochondrial fusion, both processes that are key for maintaining mitochondrial function when cells are faced with metabolic stresses.

In conclusion, the studies undertaken for this thesis advance our understanding of the mechanisms underpinning exercise-nutrient interactions, which impact on substrate utilisation and consequently exercise performance. The results from this thesis highlight the importance of CHO fuel sources for high-intensity endurance exercise and provide scientific evidence for sports nutrition guidelines, including rationale for future work in the area of substrate metabolism and performance.

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8. Chapter 8- Appendices

8.1 Appendix 1: Research Portfolio

8.1.1 Publication statements of contribution of others

1. Hawley, J.A., & **Leckey, J.J**. (2015). Carbohydrate dependence during prolonged, intense exercise. *Sports Medicine, 45,* 5-12. doi: 10.1007/s40279-015-0400-1

Contribution statement: JL was primarily responsible for the literature search, first draft of this manuscript and final proofing. JH was involved in initial concept, drafting, writing the manuscript and final proofing.

Approximate percentage contributions: J.J. Leckey 65%; J.A. Hawley 35%.

I acknowledge that my contribution to the above paper is 65%.

J.J. Leckey   Date: 09/11/2015

As principal supervisor of this project, I certify that the above contributions are true and correct:

J.A. Hawley   Date: 09/11 /2015

2. **Leckey, JJ**, Burke LM, Morton JP & Hawley JA. (2015). Altering fatty acid availability does not impair prolonged, continuous running to fatigue: Evidence for carbohydrate dependence. *J Appl Physiol*, *120(2),* 107-13.

Contribution statement: JL was primarily responsible for the conception and design, collection and assembly of data, data analysis and interpretation, drafting, revising and approval of final manuscript. LB was involved in the conception and design, data analysis and interpretation, drafting, revising and approval of final manuscript. JM was involved in the conception and design, data analysis and interpretation, drafting, revising and approval of final manuscript. JH was involved in the conception and design, financial support, data analysis and interpretation, drafting, revising and approval of final manuscript.

Approximate percentage contributions: J.J. Leckey 70%; L.M. Burke 7.5%; J.P. Morton 7.5%; J.A. Hawley 15%.

I acknowledge that my contribution to the above paper is 70%.

J.J. Leckey   Date: 15/01/2016

As principal supervisor of this project, I certify that the above contributions are true and correct:

J.A. Hawley   Date: 15/01/2016

Coauthor signatures:

L.M. Burke   Date: 15/01/2016

J.P. Morton   Date: 15/01/2016

3. **Leckey JJ,** Ross ML, Quod M, Hawley JA, Burke LM. Ketone diester ingestion impairs time-trial performance in professional cyclists. *Provisionally accepted in Frontiers in Physiology*

Contribution statement: JL was primarily responsible for the conception and design, collection and assembly of data, data analysis and interpretation, drafting, revising and approval of final manuscript. MR was involved in the conception and design, collection and assembly of data, data analysis and interpretation, drafting and approval of final manuscript. MQ was involved in the conception and design, collection and assembly of data, data interpretation and approval of final manuscript. JH was involved in the conception and design, data interpretation, drafting and approval of final manuscript. LB was involved in the conception and design, financial support, collection and assembly of data, data analysis and interpretation, drafting, revising and approval of final manuscript.

Approximate percentage contributions: J.J. Leckey 70%; M.L.Ross 10%; Quod M; 5%; J.A. Hawley 5%; L.M. Burke 10%.

I acknowledge that my contribution to the above paper is 70%.

J.J. Leckey   Date: 13/07/2017
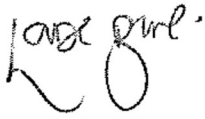
As principal supervisor of this project, I certify that the above contributions are true and correct:

J.A. Hawley

Date: 13/07/2017

Coauthor signatures:
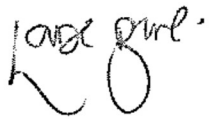
M.L. Ross

Date: 13/07/2017

M. Quod

Date: 13/07/2017

L.M Burke

Date: 13/07/2017

4. **Leckey, JJ**, Hoffman, NJ, Parr, EB, Devlin, BL, Trewin, AJ, Stepto, NK, Morton, JP, Burke, LM, Hawley, JA. (2016). High dietary fat intake increases fat oxidation and impairs skeletal muscle mitochondrial respiration in trained humans. *Submitted to the Federation of American Societies for Experimental Biology (FASEB)*

Contribution statement: JL was primarily responsible for the conception and design, collection and assembly of data, data analysis and interpretation, drafting, revising and approval of final manuscript. NH was involved in the conception and design, collection and assembly of data (including conducting mitochondrial respiration assay and analysis at Victoria University, Melbourne), data analysis and interpretation, drafting, revising and approval of final manuscript. EP and BD were involved in the conception and design, collection and assembly of data, data analysis and data interpretation and approval of final manuscript. AT was involved in the conception and design, collection and assembly of data, data analysis and interpretation, drafting, revising and approval of final manuscript. NS, JM and LB were involved in the conception and design, assembly of data, data analysis and interpretation, drafting, revising and approval of final manuscript. JH was involved in the conception and design, financial support, assembly of data, data analysis and interpretation, drafting, revising and approval of final manuscript.

Approximate percentage contributions: J.J. Leckey 70%; N.J. Hoffman 10%; E.B. Parr 2.5%; B.L. Devlin 2.5%; A.J. Trewin 2.5%; N.K. Stepto 2.5%; J.P. Morton 2.5%; L.M. Burke 2.5%; .J.A. Hawley 10%.

I acknowledge that my contribution to the above paper is 70%.

J.J. Leckey   Date: 26/09/2017

[signature]

As principal supervisor of this project, I certify that the above contributions are true and correct:

[signature]

J.A. Hawley   Date: 26/09/2017

Coauthor signatures:

[signature]
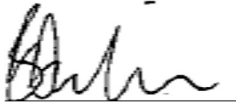
N.J. Hoffman   Date: 26/09/2017

[signature]

E.B. Parr   Date: 26/09/2017

B.L. Devlin   Date: 26/09/2017

A.J. Trewin   Date: 26/09/2017
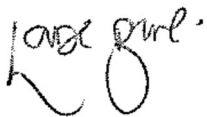
N.K. Stepto   Date: 26/09/2017

J.P. Morton   Date: 26/09/2017

L.M. Burke   Date: 26/09/2017

1. **Leckey, JJ**, Burke LM, Morton JP & Hawley JA. (2015). Altering fatty acid availability does not impair prolonged, continuous running to fatigue: Evidence for carbohydrate dependence. *J Appl Physiol*, *120(2),* 107-13.

   Presented at European College of Sports Sciences, Malmo, Sweden, June 2015.

   [Oral presentation; Awarded in Top 10 Young Investigators]

   Contribution statement: This presentation was based on the work from study 1 (author contributions listed above) and was created and delivered by JL with assistance from JH and reviewed by all authors including LB and JM.

2. **Leckey, JJ**, Hoffman, NJ, Parr, EB, Devlin, BL, Trewin, AJ, Stepto, NK, Morton, JP, Burke, LM, Hawley, JA. (2016). Does low carbohydrate or high fat availability promote fat oxidation?

   Presented at Canadian Society of Exercise Physiology, Victoria, October 2016.

   [Oral presentation]

   Contribution statement: This presentation was based on the work from study 3 (author contributions listed above) and was created and delivered by JL with assistance from JH and reviewed by all authors including NH, EP, BD, AT, NS, JM and LB.

8.2 Appendix 2: Published papers that form chapters 2, 4, 5 and 6 of this thesis.

1. Hawley, J.A., & **Leckey, J.J**. (2015). Carbohydrate dependence during prolonged, intense exercise. *Sports Medicine, 45,* 5-12. doi: 10.1007/s40279-015-0400-1.

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   https://link.springer.com/article/10.1007%2Fs40279-015-0400-1

2. **Leckey, JJ**, Burke LM, Morton JP & Hawley JA. (2015). Altering fatty acid availability does not impair prolonged, continuous running to fatigue: Evidence for carbohydrate dependence. *J Appl Physiol, 120(2),* 107-13. doi: 10.1152/japplphysiol.00855.2015

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   http://jap.physiology.org/content/120/2/107

3. **Leckey JJ,** Ross ML, Quod M, Hawley JA, Burke LM. Ketone diester ingestion impairs time-trial performance in professional cyclists. *Front Physiol, 8,* 806. doi: 10.3389/fphys.2017.00806

4. **Leckey JJ**, Hoffman NJ, Devlin BL, Parr EB, Trewin, Stepto NK, Morton JP, Burke LM, Hawley JA. (2018) High dietary fat intake increases fat oxidation and reduces skeletal muscle mitochondrial respiration in trained humans. *FASEB J,* Jan 17. Doi: 10.1096/fj.201700993R

8.3 Appendix 3: Ethical approval, participant information letters and consent forms

Study 1: Human Research Ethics Committee at Australian Catholic University Approval

Number: 2014 152V

PARTICIPANT INFORMATION LETTER

PROJECT TITLE: Carbohydrate dependence in half-marathon performance.

PRINCIPAL INVESTIGATORS: Professor John Hawley

STUDENT RESEARCHER: Jill Leckey

STUDENT'S DEGREE: PhD in Exercise and Sports Science

Dear participant,

You are invited to participate in the research project described below.

What is the project about?

The research project investigates what type of fuel the working muscles use during half-marathon races. Endurance training stimulates a variety of muscular adaptations which enhance an athlete's ability to utilise both fat and carbohydrate as energy sources during exercise. While well-trained athletes can utilise fat-based fuels during training, carbohydrate is the preferred fuel for the working muscles during races lasting 90-100 minutes. The aims of the present study are to quantify carbohydrate and fat utilisation when running to volitional fatigue at half-marathon pace and determine if blocking the use of fat-based fuel will impair performance in well-trained runners.

Nicotinic Acid (also known as niacin or vitamin B_3) will be used to block the appearance of fat in the bloodstream (process known as lipolysis). The findings of this study will be important for athletes that regularly take part in races up to 90 minutes and will help sports nutritionists provide optimal race day nutrition strategies. We hypothesise that blocking lipolysis whilst running to volitional fatigue at half-marathon pace will not affect performance.

Who is undertaking the project?

This project is being conducted by Jill Leckey and contributes towards a PhD in the area of Exercise & Sports Science at Australian Catholic University. The project is under the supervision of Professor John Hawley.

What will I be asked to do?

Preliminary testing

First you will complete a cardiovascular risk assessment questionnaire to evaluate current health status and confirm suitability to undertake physiological tests. On the basis that there is a negligible health risk, you will have your height and body mass recorded followed by completing a test to measure your maximal aerobic capacity ($\dot{V}O_2$max test). During this test speed increases every 2 minutes until volitional fatigue. This test will be completed on a motorised treadmill using online gas analysis which calculates rates of O_2 consumption, CO_2 production and substrate utilisation.

Familiarisation

You will be required to attend the ACU laboratory for one 60-minute familiarisation session, one week prior to commencing the first experimental trial. At this time, you will undertake a 5-minute self-paced warm up followed by a 10 km run on a motorised treadmill at half-marathon pace. During this time you will not receive any feedback other than elapsed distance. Inspired and expired gas will be measured using online gas analysis for five consecutive minutes at the start of this trial and every 15 minutes until completion. The recorded respiratory gases will be used to calculate substrate utilisation using validated equations. You will not have any blood taken during this session.

Experimental protocol

You will complete four experimental trials, consisting of running until volitional fatigue at your best half-marathon pace on a motorised treadmill. Two trials will be commenced in a fed state and two in an unfed state (fasted state following an 8-10 h overnight fast) and in each condition you will be administered either Nicotinic Acid (see below) or placebo given in capsules. Trials will be single blinded (you will not know which capsules or breakfast you are consuming) and each trial will be separated by ~7-10 days. Each trial will last approximately half a day (until 12:00 h).

Diet-Exercise Control

You will be asked to refrain from caffeine, alcohol, and vigorous exercise for the 24 h preceding experimental trials. You will follow a standardised high carbohydrate diet for the 24 h before each trial (8 g/kg carbohydrate, 2 g/kg protein and 1g/kg fat) that will be provided by the researcher. This will optimise carbohydrate stores in the muscle and replicate best practise for a race day. You will be free to consume water in the 24 h prior to all experimental trials.

Experimental trials

1. You will enter the ACU laboratory at 07:00 h following an overnight fast of 8-10 h. Upon arrival a cannula will be placed in the antecubital vein in the arm and a resting blood sample (6 mL) will be taken. You will be provided with a standardised breakfast (2 g/kg carbohydrate or placebo) 2 hours before the trials start. You will be blinded to which breakfast you are consuming.
2. Thirty minutes after breakfast a second blood sample (6 mL) will be taken.

3. One hour, 30 min and 1h 45 min after breakfast you will be administered either Nicotinic Acid (10 mg/kg, 5mg/kg body mass) or placebo capsules (250 mg glucose powder).
4. A third blood sample (6 mL) will be taken prior to the commencement of a 10-minute self-paced warm up.
5. You will be fitted with a heart rate monitor and following a 10 minute warm up you will commence running to volitional fatigue at your set half-marathon pace on the motorised treadmill. You will not receive any feedback other than elapsed distance. A blood sample (6 mL) will be taken immediately upon starting, every 30 min and post trials. Inspired and expired gas will be measured for 5 consecutive minutes at the start of each trial and every 20 minutes until completion and recorded respiratory gases will be used to assess substrate utilisation.
6. Carbohydrate or an identical placebo will be given orally (via a commercially available sports gel) at a rate of 60 g per hour and in all trials you can consume water.
7. Nicotinic Acid (5 mg/kg body mass) or placebo capsules (250 mg glucose powder) will be administered every 30 mins during exercise.
8. The above steps will be completed for all trials, dependent on if the trial is "fed" or "fasted".

Blood Sampling

Venous blood samples (10 x 6 mL) will be collected pre, during and post all experimental trials via a catheter placed into the antecubital vein of the arm. The total volume of blood to be extracted during each experimental trial is approximately 60 mL, making a total of 240 mL over the course of four weeks. For reference, blood donation at Red Cross is approximately 500 mL.

Nicotinic acid

In an attempt to completely suppress fat metabolism during the exercise protocol you will be orally administered Nicotinic Acid (also known as Niacin or Vitamin B_3). Nicotinic Acid is commonly used in the treatment of hyperlipidaemia, hypertriglyceridaemia and Frederickson-Lees Levy hyperlipoproteinaemia (type II, IIB, III, IV and V) and pellagra. When ingested orally, Nicotinic Acid reduces free fatty acid (a type of lipid) release from body fat into the blood stream for several hours and causes a reduction in cholesterol concentration for several days. Four to five separate doses will be administered during two of the four experimental trials. One dose of 10mg/kg body mass 1h 30 min after consuming the standardised breakfast and another 5 mg/kg body mass 15 minutes prior to the start of the warm up, then 5mg/kg every 30 minutes during the trials. A normal side effect of Nicotinic Acid administration is flushing of the skin. The primary investigator has administered Nicotinic Acid in a previously published study with no adverse side effects (Hawley *et al*. 2000). Nicotinic Acid should not be used by persons with heart or gall bladder disease, arterial bleeding or glaucoma and is contraindicated in cases of recent myocardial infarction, pregnancy and during lactation. Nicotinic Acid interacts with adrenergic blocking agents, antihyperglycaemic therapy drugs, aspirin, clonidine, colestipol, glipizide-Isoniazid, lovastatin/ pravastatin/ simvastatin, nicotine and alcohol.

Are there any risks associated with participating in this project?

There are minor risks involved in this study due to the inclusion of minor medical procedures and working at high intensities, however all risks will be minimised during each trial.

1. You will undergo medical screening through a cardiovascular risk assessment questionnaire prior to taking part in the study. You will only take part if you are well-trained with full health.
2. You will be under close supervision during all exercise trials.
3. Nicotinic Acid will be administered intermittently as this has been shown to reduce any negative circulatory effect that occurs with a single dose. Flushing of the skin can occur when administered Nicotinic Acid, this is not detrimental to health.
4. Catheterisation (for blood sampling) can be slightly uncomfortable and can lead to the possibility of bruising, infection, phlebitis and thrombophlebitis, emboli, pain, haemorrhage and extravasation. The use of sterile, disposable catheters, syringes, swabs, etc. will markedly reduce the possibility of infection caused by this procedure. Also, the use of qualified and experienced staff for all invasive procedures will reduce the likelihood of bruising as this is primarily caused by poor venipuncture techniques.
5. Researchers are aware that the laboratory standard first aid kits are located in the Exercise Metabolism Lab and these are readily accessible in the event of a medical emergency.
6. Researchers are aware of telephone locations within the building and appropriate numbers should emergency assistance be required.
7. You will be provided with safe measures to return to residence if required after the completion of trials.

What are the benefits of the research project?

1. The chance to find out your maximal aerobic capacity and receive a breakdown of the results. This provides a measure of your current fitness levels.
3. The meaning of the physiological parameters measured and how these values can be used to further improve fitness levels will be fully explained.
4. During the protocol you will consume the optimal food for race day performance, which may help enhance your future race day nutrition strategies.

Can I withdraw from the study?

Participation in this study is completely voluntary. You are not under any obligation to participate. If you agree to participate, you can withdraw from the study at any time without adverse consequences. You are encouraged to ask questions before commencing participation in this project.

Will anyone else know the results of the project?

Data will be electronically stored on a password-protected computer, a code will be used rather than your name and only the researchers will have access to the link between the code and name. Individual information will be stored for a period of five years in a protected area at ACU and only the principal investigators will have access to this information. At the conclusion of this five year period, all data will be destroyed. If you wish to gain access to your data, please contact the principal investigator. It is expected that this study will be published in an international journal relevant to nutrition and/or exercise, presented at conferences or shared with other researchers, this will always be in a form that does not identify the participants in any way.

Will I be able to find out the results of the project?

Yes, at the conclusion of the study. Your results can be discussed in confidentiality with the investigators.

Who do I contact if I have questions about the project?

First contact: Jill Leckey Phone: 0424 701 433 or Email jill.leckey@acu.edu.au
Second contact: John Hawley: Email john.hawley@acu.edu.au

What if I have a complaint or any concerns?

The study has been approved by the Human Research Ethics Committee at Australian Catholic University (approval number 2014 152V). If you have any complaints or concerns about the conduct of the project, you may write to the Research Ethics Manager at the below address:

Research Ethics Manager
Office of the Deputy Vice-Chancellor (Research)
Australian Catholic University
North Sydney Campus
PO Box 968
North Sydney NSW 2059

Any complaint or concern will be treated in confidence and fully investigated. You will be informed of the outcome.

I want to participate, how do I sign up?

Please contact Jill Leckey via phone or email if you are interested or have further questions relating to this research project. Consent forms can be returned in person or by email. Please note: both copies of the consent form must be signed and returned to the investigators.

Yours sincerely,

PRINCIPAL INVESTIGATOR

Professor John Hawley

…………………………………

STUDENT RESEARCHER

Jill Leckey

…………………………………

Consent form

Maximal exercise test to exhaustion- VO₂max

I .... have had the procedures of the exercise to be completed explained to me. Any questions I have asked have been answered to my satisfaction. I agree that the following is true:

1. I have had the exercise test to be completed explain to me thoroughly.

2. I consent to participate in the exercise test.

3. I acknowledge that:
   a) The potential benefits and risks of the exercise test have been explained to me to my satisfaction.
   b) I am able to withdraw from participation at any time and to withdraw any unprocessed data previously supplied (unless follow-up is needed for safety reasons).
   c) The exercise to be completed is for the purpose of research and/or teaching. It may not be of direct benefit to me.
   d) The security of the research data is assured during and after completion of the exercise session. Any information that may appear in publications or be provided to other researchers will not identity me.

I agree to participate in the following tests and procedures:

- One graded exercise test to volitional exhaustion on a treadmill capturing expired gases (VO_2 and VCO_2) via a mouthpiece and automated gas analyser.

NAME OF PARTICIPANT:………………………………………………………………

SIGNATURE:……………………………………………………… DATE:……………...

SIGNATURE OF INVESTIGATOR:…………………………………………………………

DATE:………………

Consent Form

TITLE OF PROJECT: Carbohydrate dependence during half-marathon performance.

PRINCIPAL INVESTIGATORS: Professor John Hawley & Dr James Morton

STUDENT RESEARCHER: Jill Leckey

I .... have read and understood the information provided in the letter to participants and I agree to participate in the study.

I acknowledge that:

1. I have received a statement explaining the test/procedures involved in this project.
2. I consent to participate in the above project and have had the details of the tests and procedures explained to me and had the chance to ask any relevant questions.
3. I agree to strictly follow the diet and exercise schedule provided by the investigators of this project and to notify the investigators should an unforeseeable event prevent my adherence to the above procedure.
4. The potential benefits and risks of the tests or procedures have been explained to me to my satisfaction.
5. I am able to withdraw from this study at any time and to withdraw any unprocessed data previously supplied (unless follow-up is needed for safety reasons).
6. The project is for the purpose of research and/or teaching. It may not be of direct benefit to me.
7. The security of the research data is assured during and after completion of the study. The data collected during the study may be published, discussed with other researchers and a report of the project outcomes will be provided to Australian Catholic University (ACU). Any information that may appear in publications or be provided to other researchers will not identity me.

   I agree to participate in the following tests and procedures:

   - One familiarisation session at the ACU St Patricks Campus lasting approximately 60 minutes, consisting of an introduction to all equipment which will be used for the testing protocol and height/body mass measurements.
   - Ingestion of provided meals for the 24 hours before and on the morning of each day of experimental testing.
   - Ingestion of provided nicotinic acid or placebo capsules on the morning of each experimental trial.

Four separate experimental trials lasting approximately half a day each at ACU St Patrick's

Campus. This includes a breakfast provided by the researcher, regular blood samples and

expired gas measurements at set intervals whilst running to volitional fatigue at half-marathon race pace.

NAME OF PARTICIPANT: ....

SIGNATURE...   DATE........................

I AM HAPPY TO BE CONTACTED BY EMAIL: Y   N

If yes please complete your email below.

Email: ……………………………………………

SIGNATURE OF PRINCIPAL INVESTIGATOR (or SUPERVISOR):....................................
DATE:………………………..

SIGNATURE OF STUDENT RESEARCHER:..
DATE:.......................……….

Study 2: Human Research Ethics Committee at Australian Institute of Sport Approval

Number: 20161005

INFORMATION TO PARTICIPANTS

Research Title: The effect of supplementation with a ketone ester on metabolism and performance of a cycling time trial simulating the 2017 World Championship course in elite road cyclists

Principal Researchers:

Dr Megan Ross, Post-Doctoral Fellow, Australian Institute of Sport, and Professor Louise Burke, AIS Sports Nutrition

We would like to invite you to participate in this collaborative research project between the AIS, OBE and Mary Mackillop Institute of Health Research. You should only participate if you want to; choosing not to take part will not disadvantage you in any way. Before you decide whether you want to take part, it is important for you to understand why the research is being done and what your participation will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information.

Aim:

The aim of this research project is to establish whether the use of a ketone supplement can provide an additional fuel source and enhance the performance of time trial cycling, using a protocol that simulates the 2017 World Championship TT course.

Benefits:

This study will

- Provide you with an opportunity to access a safe and legal supplement that has been "secretly" used by a number of professional cyclists over the past 5 years
- Provide an opportunity to collect group evidence of whether this supplement can enhance time trial performance when used under conditions simulating the 2017 World Championships course and preparation
- Provide you with an individual opportunity to commence specific practice for the 2017 World championships and collect individual experience on the use of the ketone supplement
- Allow you and the OBE cycling team to make an evidence based decision on the use of this ketone supplement

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What is involved?

You will complete two x 31 km cycling time trials on separate days, 3 d apart, each of which is a simulation of the course characteristics of the 2017 World Road Cycling Championships TT event for Elite Men and ridden on the Velotron ergometer in the AIS lab. On the day that you undertake your V02max test during this medical/testing camp, you will be given an opportunity to familiarise yourself with this bike ergometer and the course, and to fine tune the bike set up according to your preferred TT riding position.

You will be asked to complete each TT as hard as possible to simulate a race, on one occasion you will be provided with two doses of the ketone ester supplement ("Delta G"), one just before your 30 min warm up, and the other 30 min later, just before the start of the TT. On the other occasion, you will be provided with a placebo drink (matched for taste and volume) at the identical times.

With the exception of the intake of the supplement or placebo, all nutrition practices will occur according to real-life race strategies that will be standardised for each rider and repeated for each trial. Specifically, you will

- Consume a pre-race meal on the evening prior to each trial in the AIS Dining Hall based on your usual competition preparation practices. This will be supervised by your OBE chef Nicki Strobel who is contributing as a co-researcher to this project. This meal will be recorded and repeated for the subsequent trial
- Consume a pre-race breakfast in the laboratory, 2 hours prior to the start of the warm-up, based on your normal pre-race practices and supervised as above by Nicki Strobel
- Consume small amounts of a sports drink at "technically suitable" parts of the TT course, principally to achieve the benefits of the mouth rinse effect of carbohydrate and fluid

The time trial will be preceded by a standardised warm up involving a graded series of workloads. During this warm up, we will collect respiratory gases, as occurs when you undertake a Max testing protocol for the last minutes of each work stage. This will allow us to estimate the fuels you are using at different cycling intensities (and thus any changes achieved by the introduction of the ketone esters)

Prior to the commencement of the pre-race breakfast, we will insert a small tap (cannula) in your arm to allow us to take small (10 ml or 2 tsp) samples of blood at rest, before and after the consumption of the first dose of the ketone ester, and the end of the various stages of the warm up and end of the time trial. This will allow us to follow the time course of the changes

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in ketone concentrations achieved by the supplement as well as other indices of metabolism at rest and during exercise (blood glucose, insulin, free fatty acids, lactate etc). A total of 60 ml of blood (3 Tablespoons) will be collected for each trial.

Other routine measurements collected at the end of each workload and the TT include HR, ratings of Perceived Effort and gut comfort using standard AIS score sheets

Supplements/Medication use (use where appropriate)

The study will investigate whether the use of a ketone supplement, known as Delta G, which has been specially produced by a research department from Oxford University as a safe way to consume ketones, a special fuel for the muscle and brain that your body can produce under special conditions. Since it is a new supplement, there have been few studies of its effectiveness. However, it has been reportedly used by athletes from Great Britain, who were provided with special access to this product since their preparation for the London Olympic Games. The product was developed in conjunction with the US Department of Defence, and was assessed for safety for consumer use in the USA by their Food and Drug Administration. In due course, as it is produced commercially, it will be submitted to similar authorities in other countries. In the meantime, it has been investigated in a number of published safety studies, and OBE approached ASADA for confirmation that it is not considered a banned substance on the 2016 WADA Prohibited List.

The supplement currently is not listed in the AIS Sports Supplement Framework, but this study will contribute to its entry to the B Group (Supplements currently under active research) and will assist in consideration to have it moved to a more permanent category of Group A (supplements with a good evidence base) or Group C (supplements with little evidence of benefits to performance).

The product used in this study will be directly supplied by the Oxford university research team, who conduct their own tests on each batch they make to ensure that it is pure and uncontaminated.

Who we are recruiting?

We are recruiting all participants of the 2016 OBE cycling camp at the Australian Institute of Sport who are sufficiently fit and healthy to undertake to 2 x 31 km time trials within the training/medical testing camp

Adverse Effects and Withdrawal:

Confidentiality:

During and after this study, your results will identified only by subject numbers to ensure that your anonymity is ensured. The data will be pooled into group results, and you will be

provided with your own results and the group data during the camp (performance data) and afterwards (blood analyses). You may choose to share your data with other team members and personal coaches etc, but may not reveal identifiable individualised data from other cyclists without their consent.

Once the study is completed, the data will be stored on password protected laptops and hard form in locked filing cabinets. The results may be published in scientific journals and lay reports, and presented at conferences, in the format of group data and, potentially, anonymised individual responder/non-responder data.

Ethics Approval:

The study has been approved by the Australian Institute of Sport ethics committee. If you have any concerns, you can contact the secretary of the AIS Ethics Committee on 02 6214 1577.

Further information:

If you require any further information of explanation regarding your participation in this project, you are welcome to contact members of the research team: Megan Ross (megan.ross@ausport.gov.au), Louise Burke (louise.burke@ausport.gov.au) or Marc Quod (quod.greenedge@gmail.com)

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'INFORMED CONSENT' FORM (Adult)

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Research Title: The effect of supplementation with a ketone ester on metabolism and performance of a cycling time trial simulating the 2017 World Championship course in elite road cyclists

Principal Researchers: Jill Leckey, Meg Ross, Louise Burke, Marc Quod, Paolo Menaspa, Hamilton Lee, Ida Heikura, Nicki Strobel, John Hawley

This is to certify that I, _____ hereby agree to participate as a volunteer in a scientific investigation as an authorised part of the research program of the Australian Sports Commission under the supervision of Megan Ross and Louise Burke.

The investigation and my part in the investigation have been defined and fully explained to me by _____ and I understand the explanation. A copy of the procedures of this investigation and a description of any risks and discomforts has been provided to me and has been discussed in detail with me.

- I have been given an opportunity to ask whatever questions I may have had and all such questions and inquiries have been answered to my satisfaction.
- I understand that I am free to deny any answers to specific items or questions in interviews or questionnaires.
- I understand that I am free to withdraw consent and to discontinue participation in the project or activity at any time, without disadvantage to myself.
- I understand that I am free to withdraw my data from analysis without disadvantage to myself.
- I understand that any data or answers to questions will remain confidential with regard to my identity.
- I certify to the best of my knowledge and belief, I have no physical or mental illness or weakness that would increase the risk to me of participating in this investigation.
- I am participating in this project of my (his/her) own free will and I have not been coerced in any way to participate.
- I have read and understand the product and policy information provided to me on surrounding the use of supplements/medications within the study (where applicable)

__Privacy Statement__: The information submitted will be managed in accordance with the ASC Privacy Policy.

□ *I consent to the ASC keeping my personal information.*

Signature of Subject: _____   Date: ___/___/___

I, the undersigned, was present when the study was explained to the subject/s in detail and to the best of my knowledge and belief it was understood.

Signature of Researcher: _____   Date: ___/___/___

Study 3: Human Research Ethics Committee at Australian Catholic University Approval

Number: 2014 152V

Participant information letter

Title of project: Effects of low carbohydrate availability with high protein or high fat on the

response to training and performance.

Who is undertaking the project?

Student researcher: Jill Leckey
Jill is conducting this study as part a PhD at Australian Catholic University. Jill has lead several research studies on sports nutrition and metabolism which have been published in scientific papers.

Principal investigator: Professor John Hawley
The project is under the supervision of Professor John Hawley. John is currently Director for the Centre of Exercise and Nutrition at the Mary MacKillop Institute of Health Research, Australian Catholic Uni and has published over 200 scientific papers within the area of sports nutrition and metabolism.

You are invited to participate in the research project described below.

What is the project about?

Endurance performance lasting >90 min is limited by carbohydrate stores. Therefore for the past few decades' researchers have proposed ways to increase the use of fat as a fuel source, reducing reliance on carbohydrate sources during endurance events and 'sparing' muscle glycogen. Recent studies have focused on high fat, low carbohydrate diets, which have become increasingly popular amongst athletes, with the expectation of a greater response to training and improved performance. High fat, low carbohydrate diets (~ 65% energy intake from fat) for up to 2 weeks have been shown to increase the use of fat as a fuel source during low to moderate intensity cycling however an improvement in cycling performance is yet to be reported. Another nutritional strategy prescribed to increase the use of fat during training is a high protein, low carbohydrate diet, otherwise known as 'train low' (i.e. with low carbohydrate stores). Although both strategies increase the use of fat stores, the complex muscular responses and mechanisms behind these adaptations have yet to be reported

What will I be asked to do?
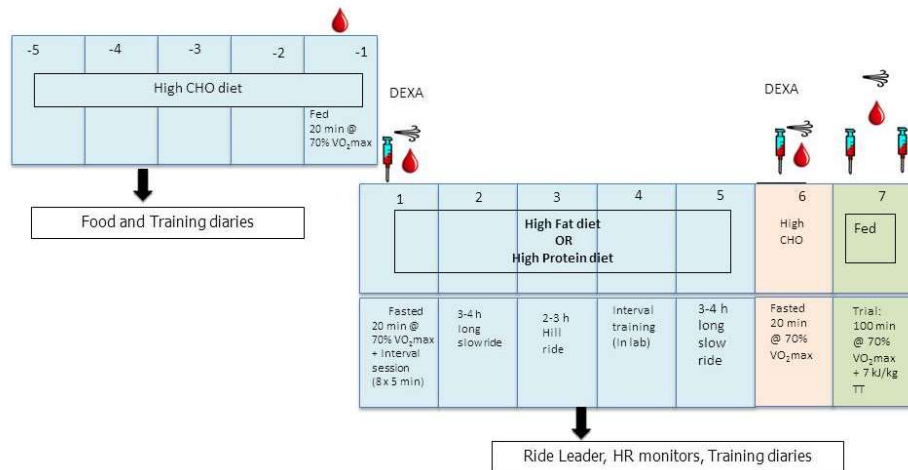
Are there any risks associated with participating in this project?

There are minor risks involved in this study due to the inclusion of minor medical procedures and working at high intensities, however all risks will be minimised during each trial.

1. You will undergo medical screening through a cardiovascular risk assessment questionnaire prior to taking part in the study. You will only take part if you are well-trained with full health.
2. You will be under close supervision during all exercise trials.
3. Catheterisation (for blood sampling) can be slightly uncomfortable and can lead to the possibility of bruising, infection, phlebitis and thrombophlebitis, emboli, pain, haemorrhage and extravasation. The use of sterile, disposable catheters, syringes, swabs, etc. will markedly reduce the possibility of infection caused by this procedure. Also, the use of qualified and experienced staff for all invasive procedures will reduce the likelihood of bruising as this is primarily caused by poor venepuncture techniques.
4. The amount of radio-labelled glucose tracer being infused is the minimal level practicable. The dose is considerably lower than the dose constraint listed in the Code of Practice by ARPANSA.
5. Muscle biopsies can result in a small amount of bruising, swelling, and bleeding and there is a low risk of nerve injury. All biopsies will be completed following the use of a small amount of local anaesthetic, sterile needles, sterile consumables and an extremely experienced doctor (Dr. Andrew Garnham) to reduce any risk of feeling uncomfortable/pain. Andrew has previously completed 1000s of muscle biopsies.
6. Researchers are aware that the laboratory standard first aid kits are located in the Exercise Metabolism Lab and these are readily accessible in the event of a medical emergency.
7. Researchers are aware of telephone locations within the building and appropriate numbers should emergency assistance be required.

What are the benefits of the research project?

1. The opportunity for you to find out your maximal aerobic capacity and peak power output and receive a breakdown of the results. This provides a measure of current aerobic fitness levels.
2. The meaning of the physiological parameters measured and how these values can be used to further improve fitness levels will be fully explained.
3. During the protocol a dietician will make up your carbohydrate loading diet for day 6, this can be used prior to other training sessions.
4. Estimated muscle glycogen utilisation during a 2 h submaximal ride.

Can I withdraw from the study?

Participation in this study is completely voluntary. If you agree to participate, you can withdraw from the study at any time without adverse consequences. You are encouraged to ask questions before commencing participation in this project.

Will anyone else know the results of the project?

Data will be electronically stored on a password-protected computer, a code will be used rather than your name and only the researchers will have access to the link between the code and name. Individual information will be stored for a period of five years in a protected area at ACU and only the principal investigators will have access to this information. At the conclusion of this five year period, all data will be destroyed. If you wish to gain access to your data, please contact the principal investigator. It is expected that this study will be published in an international journal relevant to nutrition and/or exercise, presented at conferences or shared with other researchers, this will always be in a form that does not identify the participants in any way.

Will I be able to find out the results of the project?

Yes, at the conclusion of the study. Your results can be discussed in confidentiality with the investigators.

What if I have a complaint or any concerns?

The study has been approved by the Human Research Ethics Committee at Australian Catholic University (approval number 2015-305H). If you have any complaints or concerns about the conduct of the project, you may write to the Research Ethics Manager at the below address:

Research Ethics Manager
Office of the Deputy Vice-Chancellor (Research)
Australian Catholic University
North Sydney Campus
PO Box 968
North Sydney NSW 2059

Any complaint or concern will be treated in confidence and fully investigated. You will be informed of the outcome.

Who do I contact if I have questions about the project?

First contact: Jill Leckey   Phone: 0424 701 433   Email jill.leckey@acu.edu.au
Second contact: John Hawley: Email john.hawley@acu.edu.au

I want to participate, how do I sign up?

Please contact Jill Leckey via phone or email if you are interested or have further questions relating to this research project. Consent forms can be returned in person or by email. Please note: both copies of the consent form must be signed and returned to the investigators.
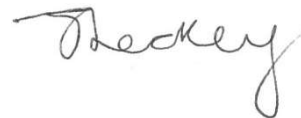
Yours sincerely,

PRINCIPAL INVESTIGATOR   **STUDENT**
RESEARCHER

Professor John Hawley   Jill Leckey

Details of study participation

Title of project: Effects of low carbohydrate availability with high protein or high fat on the response to training and performance.

Preliminary testing:

- First you will complete a cardiovascular risk assessment questionnaire to evaluate current health status and confirm suitability to undertake physiological tests.
- On the basis that there is a negligible health risk, you will have your height and body mass recorded followed by completing a test to measure your maximal aerobic capacity ($\dot{V}O_2$max test) and Peak Power Output (PPO).
- Prior to completing the test you will complete a 15-minute self-paced warm-up at a light intensity. A VO_2max test is an incremental exercise test therefore workload will increase every 150 s until you can no longer keep up the required pedal cadence (above 60 rev/min). Peak power output will then be determined using a validated equation. During this exercise test you will wear a mouthpiece so that expired gas can be collected using online gas analysis then we can calculate rates of O_2 consumption, CO_2 production and the energy source used to fuel the exercise.

Day 1 & Day 6 DEXA:

- The first is a whole body x-ray using a dual-energy x-ray absorptiometry (DXA) machine to give estimates of your body composition (fat and lean mass). The scan will take ~15 minutes. The machine uses **small doses (<1% of the yearly radiation dose) of radiation** to estimate tissue density. This test **requires you to be fasted** with no food, fluid or exercise/activity prior to the test and requires you to wear light clothing with no metal items (i.e. zips, domes, clips, underwire etc). Please check with the research staff if you are unsure.

Dietary control:

You will complete an 8-day supervised feeding protocol. During this time a dietician accredited by Sports Dieticians Australia will provide you with all meals and snacks. For the first three days of the study (-5 to -3), you will be provided with a food top up to ensure your carbohydrate intake is high. For days -2 and -1, you will be provided a high carbohydrate diet containing 70% energy from carbohydrate. For days 1-5 the diet will either be a high-fat, low carbohydrate diet containing 70% fat, 15% carbohydrate, 15% protein (including 50 g fibre) or a high protein, low carbohydrate diet consisting of 70% protein, 15% carbohydrate, 15% fat (including 50 g fibre) (for a 70 kg person this equates to ~4000 kcal). For day 6 you will follow a carbohydrate

diet to increase muscle glycogen stores consisting of 10 g/kg BM carbohydrate (CHO), 2 g/kg BM protein and 1 g/kg BM fat (for a 70 kg person this equates to ~3400 kcal). During these 8 days you will be required to abstain from caffeine and alcohol. There will be a minimum of 2 weeks break period between the two different diets.

Diet control day 7, experimental trial day:

- You will enter the lab following an overnight fast and will be provided a standardised jelly and 600 ml fluid.
- Following the trial you will be provided a small snack prior to leaving the lab (i.e. up & go chocolate milk).
- You will be free to consume water ad libitum during all training sessions and the exercise trial.

Exercise control:

You will complete a 7-day supervised training protocol which has been used in research previously. You will complete sessions on days -1, 1, 4 and 6 in the ACU lab; other training sessions will be self-directed or completed with a ride leader. During the labs sessions, expired gas will be collected via a mouthpiece.

Experimental ride (day 7):

1. On the morning of day 7 you will report to the lab at 07:00 h after a 10-12 h overnight fast.
2. A cannula will be inserted into the antecubital vein of the left arm and a baseline blood sample (10 mL) will be taken. Following each blood-draw the cannula will be flushed with saline (5 mL NaCl) to keep the vein patent.
3. A small amount of local anaesthetic will then be injected under the skin of the outer thigh and a muscle biopsy will be taken (described later).
4. You will then ingest a standardised breakfast and rest for 120 min before the trial commences.
5. A blood sample (10 mL) will be taken at 30 min post breakfast.
6. At 100 min post breakfast, you will be given a single bolus infusion of trace amounts of 0.37 Mbq U-14C-labeled glucose (10 µCi) in 10 mL of 0.9% saline for the determination of the rates of blood glucose oxidation (Used previously by Arkinstall et al., 2001).
7. At 100 min following breakfast, your body mass will be recorded, a heart rate monitor will be fitted and you will get ready to start the exercise.
8. A blood sample will be taken whilst you are sitting on the bike immediately pre-exercise.
9. You will then ride on a cycle ergometer for 100 min at 70% VO_2max.
10. Blood samples will be collected every 20 min (10 mL) and RPE (scale 6-10) and heart rate will also be recorded at this time.
11. Expired gas (labeled $^{14}CO_2$) will be collected every 20 min for a period of 4 min via a mouthpiece connected to a 2-litre anaesthesia bag.
12. You can consume as much water as you want throughout the exercise.
13. Immediately upon exercise completion, you will have another muscle biopsy and blood sample (10 mL).
14. You will complete a 7 kJ/kg time trial, which will take approx. 30-35 min.

15. The above steps will be completed for day 7 of both dietary interventions.

Radio-labelled glucose tracer:

Twenty minutes before you start exercising you will be infused with a single bolus infusion of 0.37 MBq U-14C-labeled glucose (10 uCi) in 10 mL of 0.9% saline. The expected radiation dose for this procedure is approximately 0.16 mSv, which is well below the level of background radiation received by every person from natural sources (2-3 mSv) and a small fraction of the 1 mSv yearly limit afforded to members of the public (in addition to background radiation).

The storage, preparation and disposal of the radio-labelled tracer will be carried out in accordance with the Australian Radiation Protection and Nuclear Safety Agency (ARPANSA) code of practice. The student researcher Jill Leckey who has attended the lab safety radiation course by ANSTO will carry out the infusion. Analysing the expired breath of participants will attain a measure of radioactivity.

Radio-isotopes have been used previously by the chief investigator to investigate carbohydrate use during exercise.

Blood sampling and analysis:

Day 1, 4 and 6: Venous blood samples (10 mL) will be collected on days 1 and 6 (see figure 1) into collection tubes. Samples will be collected via a venepuncture into the antecubital vein of the arm. A total of 20 mL will be collected across the 2 days. On day 4, a finger prick blood sample will be collected pre and post the exercise session (0.2 mL) and analysed immediately.

Day 7: Venous blood samples will be collected into collection tubes via a catheter placed into the antecubital vein of the arm. A total of 90 mL will be collected on day 7.

The total volume of blood to be extracted during each 7-day protocol is approximately 130 mL, making 260 mL across both dietary interventions. For reference, blood donation at Red Cross is approximately 500 mL.

Muscle Biopsies:

A total of four biopsies will be obtained during each 7-day protocol. The muscle biopsy will be obtained from the outer thigh by Dr. Andrew Garnham. In preparation for a biopsy, a small amount of local anaesthetic is injected under the skin, which may result in a mild burning sensation while the fluid is injected. A small, 4-5 mm incision is then be made into the skin to create an opening for the biopsy needle. There is often a small amount of bleeding from the incision; however this bleeding is generally minimal. The biopsy needle is then inserted through the incision site. You may feel the sensation of deep pressure in the biopsy site, and on some occasions this is moderately painful. However, the discomfort very quickly passes and you are capable of performing exercise and daily activities within minutes. There may also be some minimal bleeding when the needle is removed which may require the application of pressure for a few minutes. When having a muscle biopsy there is a low risk of nerve injury and low-medium risk of swelling/bruising.

After each biopsy, the incision will be closed with sterile tape and wrapped with a bandage. Once the anaesthetic freezing wears off, your leg may feel tight and often there is the sensation of a deep bruise. Painkillers such as paracetamol (e.g. Panadol) or Ibuprofen (e.g. Advil) are acceptable to use if you experience pain associated with the biopsy. Periodically applying an ice pack to the biopsy site the following day reduces any local swelling and/or residual soreness. Your leg may feel uncomfortable the following day when walking down stairs and to a lesser extent activities that involve forceful movements of the abdomen. To allow the incisions to heal properly and minimize any risk of infection, you should avoid prolonged submersion in water for 4 days. Daily showers are acceptable, but baths, swimming, saunas etc. should be avoided for at least 4 days following the biopsy. You are encouraged to visit Dr. Andrew Garnham 7-10 days following the experimental trial day to check the biopsy safely and re-apply dressings/ bandages should it be required.

Consent form- Researcher copy

Title of project: Effects of low carbohydrate availability with high protein or high fat on the

response to training and performance.

Principal investigators: Professor John Hawley

Student researcher: Jill Leckey

I .... have read and understood the information provided in the letter to participants and I agree to participate in the study.

1. I have received a statement explaining the test/procedures involved in this project.
2. I consent to participate in the above project and have had the details of the tests and procedures explained to me and had the chance to ask any relevant questions.
3. I agree to strictly follow the diet and exercise schedule provided by the investigators of this project and to notify the investigators should an unforeseeable event prevent my adherence to the above procedure.

I acknowledge that:
4. The potential benefits and risks of the tests or procedures have been explained to me to my satisfaction.
5. I am able to withdraw from this study at any time and to withdraw any unprocessed data previously supplied (unless follow-up is needed for safety reasons).
6. The project is for the purpose of research and/or teaching. It may not be of direct benefit to me.
7. The security of the research data is assured during and after completion of the study. The data collected during the study may be published, discussed with other researchers and a report of the project outcomes will be provided to Australian Catholic University (ACU). Any information that may appear in publications or be provided to other researchers will not identity me.
8. I have been informed to retain the information sheet which includes the radiation dose for at least 5 years following the study so that it can be provided in future research projects involving exposure to radiation.

I agree to participate in the following tests and procedures:

- DEXA to measure body composition.
- Ingestion of all provided meals during the experimental testing period.

- All training sessions required.
- All blood samples and muscle biopsy samples across the two 12-day periods.
- On day 7 completing the experimental trial lasting approximately 5 hours each at ACU St Patrick's Campus, inclusive of a provided breakfast, 2 muscle biopsies, 9 blood samples at regular intervals and the infusion of trace amounts of U-^{14}C-labeled glucose (10 uCi) in 10 mL of 0.9% saline.

NAME OF PARTICIPANT: ....

SIGNATURE ....

DATE ....

SIGNATURE OF PRINCIPAL INVESTIGATOR (or SUPERVISOR):......................................

DATE:………………………..

SIGNATURE OF STUDENT RESEARCHER: ....

DATE:.......................……….

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