DIETARY INFLUENCES ON EXERCISE METABOLISM, HEALTH AND ENDURANCE PERFORMANCE

By

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Abstract

The capacity to oxidise lipid whilst physically active has been associated with markers of metabolic health and exercise performance. In a group of 305 active men and women, this thesis observed a substantial 6-fold inter-individual variability in the capacity to oxidise lipid during exercise, and explains 46% of the variability. This was largely attributed to aerobic capacity, biological sex, self-reported physical activity level and body composition, with new information provided to show that the dietary intake of carbohydrate and fat is also a significant contributor (~3%) to the explained inter-individual variability.

Prior research in men, demonstrates that high fat, low carbohydrate diets enhance lipid oxidation during exercise. This thesis demonstrates that, like men, women respond to short-term (5 day) high fat, low carbohydrate diets by considerably (33%) increasing lipid oxidation during exercise. Further, by using a short term hypercaloric 'fat supplementation' without carbohydrate restriction diet, which did not alter lipid oxidation during exercise, this thesis suggests that carbohydrate restriction, not increased fat intake, drives the increased lipid oxidation observed during high fat, low carbohydrate diets. Finally, short-term dietary fat manipulation appears to have minimal impact upon markers of metabolic health or endurance exercise performance in the well-trained women studied.

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Abbreviations

A/LB FM, the abdominal to lower body fat mass ratio; ADP, adenosine diphosphate; AMP, adenosine monophosphate; AMPK, AMP-activated protein kinase; AST, aspartate aminotransferase; ATGL, Adipose triglyceride lipase; ATP, adenosine triphosphate; BCOAD, branched-chain 2-oxo acid dehydrogenase; Beta HAD, Beta-hydroxyacyl-CoA dehydrogenase; BM, Body Mass; BMI, Body Mass Index; Ca^{2+}, calcium ion; CAT, acyl carnitine translocase; CHO, Carbohydrate; CHO-OX, carbohydrate oxidation; CTP1, Carnitine palmitoyl transferase I; CPTII, carnitine palmityltransferase II; DAG, diacylglecerol; DXA, Dual-Energy X-ray Absorptiometry; EDTA, ethylenediamine tetra-acetate; EE, energy expenditure; EI, Energy Intake; FA, fatty acids; FABp, FatMax, Exercise Intensity Eliciting MFO; fatty acid binding protein; FAT/CD36, Fatty acid translocase/cluster of differentiation 36; FABPc - cytosol fatty acid binding protein; FABPpm, plasm membrane fatty acid binding protein; FAT-OX fat oxidation; FFM, Fat Free Mass; FM, Fat Mass; FOL, follicular; GS, glycogen synthase; GLUT4, glucose transporter 4; HC, Hormonal Contraceptive; HCLF; High carbohydrate low fat diet; HF, High Fat diet; HFLC, High fat low carbohydrate diet; HKII, hexokinase II; HSL, Hormone sensitive lipase; LH, Luteinising hormone; LPL, lipoprotein lipase; LUT, luteal, MAG, Monoacylglycerol lipase; MET, metabolic equivalent; MFO, Maximal Rate of Fat Oxidation;; mRNA, messenger ribonucleic acid; mtGPAT, mitochondrial glycerol phosphate acyl transferase; NEFA, non-esterified fatty acids; NHF, normal diet fat supplemented; NORM, Normal diet, NRF1, nuclear respiratory factor 1; PDK4, pyruvate dehydrogenase kinase 4; PFK, phosphofructokinase; PGC-1alpha, peroxisome proliferator activated receptor gamma coactivator-l alpha; PPAR, peroxisome proliferator activated receptor; PROGEST, progesterone only contraceptive; Ra, rate of appearance; Rd, rate of disappearance; REG,

Regularly menstruating; RER, Respiratory Exchange Ratio; RMR, Resting Metabolic Rate; RQ, respiratory quotient; SREBP1, Sterol regulatory element-binding protein 1; SRPAL, Self-Reported Physical Activity Level; TAG, Triacylglycerol; TCA cycle, tricarboxylic acid cycle; $\dot{V}O_2$max, Maximal Aerobic Capacity; WC, Waist Circumference.

1 THESIS OUTLINE

This thesis explores the impact of dietary intake on substrate oxidation during exercise. The thesis begins with a General Introduction (Chapter 2) to the topic areas pertinent to the overall thesis followed by sections that discuss in more detail key areas in the current literature that warranted further investigation. The General Methods section (Chapter 3) describes the principles behind the main methods employed during the conduct of the research presented in this thesis, highlighting key strengths and limitations of the approaches taken. Chapter 4, the first experimental chapter of the thesis, presents a large cross sectional study that was conducted to investigate the determinants of the frequently reported large inter-individual variability in fat oxidation during exercise, with a particular focus on the role of nutrition. This Chapter is spilt into 3 sections, the first of which (Section 4.1) is in the form of a manuscript currently under review for publication using the main data collected from this study. Using the same data set but split by sex, Section 4.3 explores whether the determinants of fat oxidation differ in men and women. Section 4.2 focused solely on the women from the same data set, and sought to explore the role of menstrual cycle and hormonal contraceptive use on fat oxidation during exercise. The conclusions drawn from Chapter 4 informed the design of the study described in Chapter 5, which presents the second experimental chapter of the thesis. This study sought to investigate the influence of varying short-term (i.e., 5 days) dietary fat intake on exercise metabolism and performance in well-trained female runners. The thesis ends with a General Discussion and Conclusion (Chapter 6) which discusses the new contributions made in light of the aims and

objectives of the thesis, as well as their relevance, limitations and implications for future research.

2 GENERAL INTRODUCTION

2.1 Introduction

During exercise energy requirements increase many-fold and this metabolic demand is primarily met through an increased oxidation of carbohydrates and lipids, with the capacity to utilise the latter implicated as being important for both metabolic health and exercise performance. For instance, insulin sensitivity (a marker of metabolic health) has been associated with the maximal attainable rate of fat oxidation (MFO) during exercise in young healthy men (Robinson et al. 2015). From an exercise performance perspective, increasing lipid oxidation during exercise is desirable in many endurance-based sports as a way to delay the onset of fatigue through minimising the depletion of the body's limited endogenous carbohydrate store (Jeukendrup, Saris, and Wagenmakers 1998). Thus, there is relevance from the perspective of the general population and those looking to improve exercise performance in understanding the regulation of fat oxidation and ways to enhance it.

Interestingly, the relative proportion of fat and carbohydrate oxidised to meet energy requirements during low-moderate intensity exercise is highly variable between individuals (Goedecke et al. 2000). Indeed, MFO has been shown to range over 5 fold between individuals (0.18-1.01g/min) (Achten and Jeukendrup 2003; Venables, Achten, and Jeukendrup 2005). Previously, 35% of this large inter-individual variability in MFO was explained by self-reported physical activity level, $\dot{V}O_2$max, body composition and biological sex (Venables, Achten, and Jeukendrup 2005). Dietary intake can affect substrate oxidation during low-moderate intensity exercise (Helge, Richter, and Kiens 1996; Jansson 1982),

although the extent to which commonly consumed habitual diets contribute to the frequently observed inter-individual variability in fat oxidation has not been comprehensively addressed. A more thorough understanding of the factors responsible for inter-individual variability in fat oxidation may allow for their superior prescription and manipulation, with the potential to improve metabolic health and/or exercise performance.

Numerous sex based differences have been described in exercise metabolism and substrate oxidation, for instance, compared to men, women oxidise a greater proportion of fat to carbohydrate during exercise (Tarnopolsky et al. 1990; Tarnopolsky et al. 1995). Sex based differences in anatomy and physiology account for some differences as does the markedly greater circulating concentration of the hormone oestrogen in women, with this hormone shown to augment whole body fat oxidation during exercise (Maher, Akhtar, and Tarnopolsky 2010), and regulate the transcription of genes involved with IMTAG storage which can then further modulate substrate oxidation (Fu et al. 2009). With these numerous sex based differences in physiology and metabolism there is the potential for a sex difference in the response to the effect of dietary intake and substrate oxidation. Despite numerous studies in men of the impact of manipulating dietary fat intake on metabolism and exercise performance, there are few, well controlled (dietary control) studies characterising the response in women.

The following section introduces the relevant literature related to fuel use, its variability and the factors influencing fuel use during exercise with a particular focus on diet and biological sex. In doing this, key knowledge gaps in the field are highlighted that underpin the need for

new research, some of which has been undertaken during the course of this doctoral research and is presented in this thesis.

2.2 Fuels for exercise

2.2.1 Storage, mobilisation & utilisation

Adenosine Tri-Phosphate (ATP) is the chemical energy source, the hydrolysis of which releases energy to support energy consuming processes such as muscular contraction. The cellular levels of ATP however are very small and must be constantly re-synthesized in an energy consuming process, which during exercise of over around 60secs is primarily supplied through the oxidation of fatty acids and carbohydrates. The following section briefly overviews the storage, mobilisation and utilisation of these major energy substrates. The interested is referred to recent articles for further detail (Spriet 2014; Van Hall 2015).

Exercise results in an increase in the rate of delivery and muscle extraction of plasma glucose, with this tightly regulated so that the increased rate of extraction is matched by a reciprocal rise in hepatic glucose production (Ahlborg et al. 1974). The liver is able to sustain an increased output of glucose for 60-90mins at moderate-to-hard exercise intensities (60-70% $\dot{V}O_2$max) (Ahlborg and Felig 1982; Coyle et al. 1986) through a combination of both glycogenolysis and gluconeogenesis (Wahren et al. 1971; Trimmer et al. 2002), although this markedly diminishes the liver glycogen content (Stevenson et al. 2009) unless exogenous carbohydrate is also ingested (Gonzalez et al. 2015). Glycogenolysis of the hepatic glycogen stores is the predominant (~70-80%) source of glucose production during exercise (Trimmer et al. 2002). If this small (~80g) store were the only source of glucose production however, it

would be completely depleted in under 3hrs of exercise at 65% $\dot{V}O_2$max, and so the ability to maintain normal glycaemia calls upon an increased contribution of gluconeogenesis to overall hepatic glucose production (Trimmer et al. 2002). Under such circumstances, hepatic glucose production is facilitated by increases in the gluconeogenic precursor pool of both lactate, alanine and glycerol (Friedlander et al. 1999; Chung et al. 2015). The change in the gluconeogenic precursor pool is dependent on both the exercise intensity and duration (Friedlander et al. 1999). Although there is an increase in the absolute rate of gluconeogenesis with exercise intensity, the relative contribution to overall hepatic glucose production falls or changes minimally, with glycogenolysis the dominate (~70-80%) source of glucose production (Trimmer et al. 2002).

Glucose is also stored within skeletal muscle in the form of glucosyl units in glycogen. Although the size of the muscle glycogen store is malleable and is particularly sensitive to dietary carbohydrate intake and physical activity level (Mc Inerney et al. 2005; Bergstrom et al. 1967), values of ~130mmol/ kg wet wt (560mmol/kg dry wt) are typical on a diet containing 50% carbohydrates (Costill et al. 1981; Sherman et al. 1981). The presence of glycogen localised within skeletal muscle offers a readily available glucose source for exercise without the mobilisation and transport limitations of liver-derived glucose. At exercise intensities between ~65-80% $\dot{V}O_2$max muscle glycogen utilisation is substantial, with the onset of fatigue often associated with muscle glycogen depletion (Ahlborg et al. 1967; Bergstrom et al. 1967; Hermansen, Hultman, and Saltin 1967). Collectively, the onset of fatigue during prolonged exercise of 65-80% $\dot{V}O_2$max has been attributed to reductions in liver and/or muscle depletion often manifesting as inability to maintain glucose and/or

carbohydrate oxidation at sufficiently high rates to for the required workload (Coggan and Coyle 1987).

Fatty acids are primarily stored in subcutaneous and deep visceral adipose tissue as triacylglycerol (TAG), with smaller quantities also found within or between muscle fibres as intra or inter muscular triacylglycerol (IMTAG) respectively. IMTAG content is variable between individuals ranging between 2-10mmol/kg wet wt and as discussed later is related to a number of factors including dietary fat intake, sex and training status (Van Loon 2004). The sympathetic nervous system is the major positive regulator of adipose tissue lipolysis during exercise, through the stimulatory release of catecholamines, that phosphorylate perilipins resulting in a co-ordinated catabolism of TAGs initially through the rate limiting action of adipose triglyceride lipase (ATGL) followed by hormone sensitive lipase (HSL) and then monoacylglycerol lipase (MAGL) splitting the final acyl group liberating glycerol and non-esterified fatty acids (NEFAs) (Aon, Bhatt, and Cortassa 2014; Zimmermann et al. 2004). Insulin has the opposing action and acts to inhibit lipolysis by preventing the phosphorylation and activation of HSL (Langin, Holm, and Lafontan 1996). Other processes particularly pertinent during exercise such as an increase in glycolytic flux (Boyd et al. 1974) leading to lactate accumulation in plasma can also inhibit the rate of lipolysis through the G protein coupled receptor GPR81 (Liu et al. 2009) thus limiting plasma NEFA availability for oxidation (Frayn 2010).

NEFAs liberated from adipose tissue TAG lipolysis are transported in the circulation via albumin to skeletal muscle. Lipids are also present in much smaller quantities in the bloodstream, either circulating bound to albumin as NEFA or incorporated as TAG and transported within the core of the different types of lipoprotein particles (chylomicrons, very-low [VLDL], low [LDL], and high [HDL] density lipoproteins), which are hydrolysed by lipoprotein lipase (LPL) located in the capillary endothelium of skeletal muscle. The resulting NEFAs released from these lipoprotein particles are only thought to provide a small (<10%) contribution to total fat oxidation during exercise (Havel, Pernow, and Jones 1967). Plasma NEFAs taken up by myocytes are transported across the cytoplasm of the cell before crossing the outer and inner mitochondrial membrane to reach the mitochondrial matrix for beta-oxidation, with anyone or a combination of these steps potentially limiting the rate of fat oxidation (Van Hall 2015). In a similar manner to the myocellular stores of glycogen, with their close proximity to the site of oxidation, IMTAG provide an immediate substrate source for oxidation, before the slower mobilisation and delivery of adipose derived NEFA.

2.3 Factors affecting fuel use during exercise

2.3.1 Intensity

The intensity of exercise is arguably the most influential factor determining the absolute and relative contribution of the different fuels oxidised during exercise. For clarity, throughout this thesis, exercise intensity will be either quantified as a relative proportion of aerobic capacity (percentage of $\dot{V}O_2max$) or by a qualitative descriptor, specifically; mild/low (<45% $\dot{V}O_2max$), moderate (50-65% $\dot{V}O_2max$), moderate to hard (65-75% $\dot{V}O_2max$), hard /

demanding (>75% $\dot{V}O_2$max). When considered on a whole-body level, the relative contribution of fat and carbohydrate to total energy expenditure as a function of exercise intensity has been described as the cross-over concept (Brooks and Mercier 1994). This describes a gradual decrease in the relative contribution of fat to energy expenditure from ~60% at rest, declining to <30% at moderate intensities and declining more so during hard intensity exercise down to <10%, with the relative contribution of carbohydrate following the opposite pattern. The cross-over point is the exercise intensity at which energy derived from carbohydrate based fuels predominates over that derived from lipids, typically occurring at low-moderate intensities, with further increases in exercise intensity beyond this point prompting a relative increment in energy from carbohydrate and decrement in energy from fat oxidation (Brooks and Mercier 1994).

As exercise intensity increases and so the overall demand for energy increases, the absolute rate of fat and carbohydrate oxidation must also change to meet the energy demands. Typically the absolute rate of fat oxidation as a function of exercise intensity follows an inverted U pattern, with rates of fat oxidation increasing from rest to a peak around 50-60% $\dot{V}O_2$max, termed the maximal rate of fat oxidation (MFO), upon which it declines with any further increase in exercise intensity to negligible levels >85% $\dot{V}O_2$max (see Figure 2-1 & Figure 2-2) (Jeukendrup and Wallis 2005; Venables, Achten, and Jeukendrup 2005; Achten, Gleeson, and Jeukendrup 2002; Achten and Jeukendrup 2003). On the other hand, the rate of carbohydrate oxidation continues to rise from resting values in line with the increasing workload, becoming the dominant source for oxidation from around 50-60% $\dot{V}O_2$max, with the rate rising exponentially thereafter with further increases in exercise intensity (van Loon

et al. 2001; Romijn et al. 1993; Achten, Gleeson, and Jeukendrup 2002; Achten and Jeukendrup 2003; Achten, Venables, and Jeukendrup 2003; Venables, Achten, and Jeukendrup 2005; Rosenkilde et al. 2010; Stisen et al. 2006; Cheneviere et al. 2011; Nordby, Saltin, and Helge 2006). The greater biochemical efficiency of carbohydrate oxidation over lipid is represented by the ~7.7% greater energy liberated per unit of oxygen consumed (Brooks 2012). This makes carbohydrate the preferred substrate when the rate of energy demand is high and/or oxygen supply to the muscle is compromised, such as during intense exercise or in hypoxia (Peronnet et al. 2006).

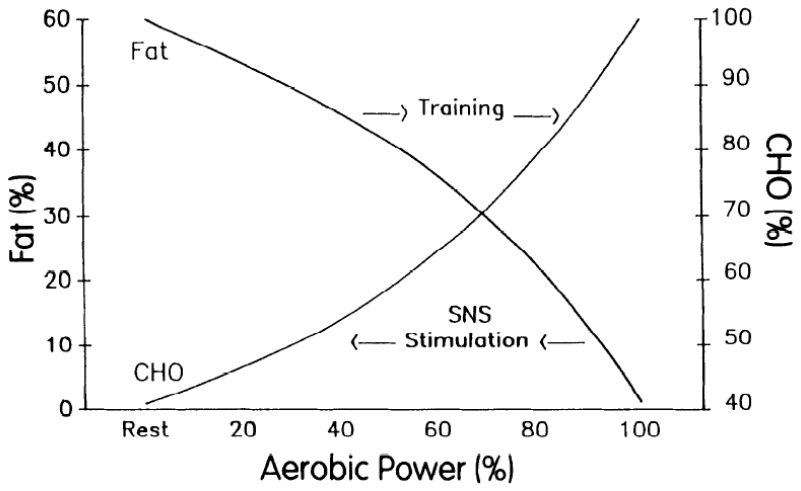

Figure 2-1 The "cross-over concept" Showing the relative increase in energy derived from carbohydrate utilisation and decline in energy from the oxidation of fat as a function of exercise intensity, taken from (Brooks and Mercier 1994).

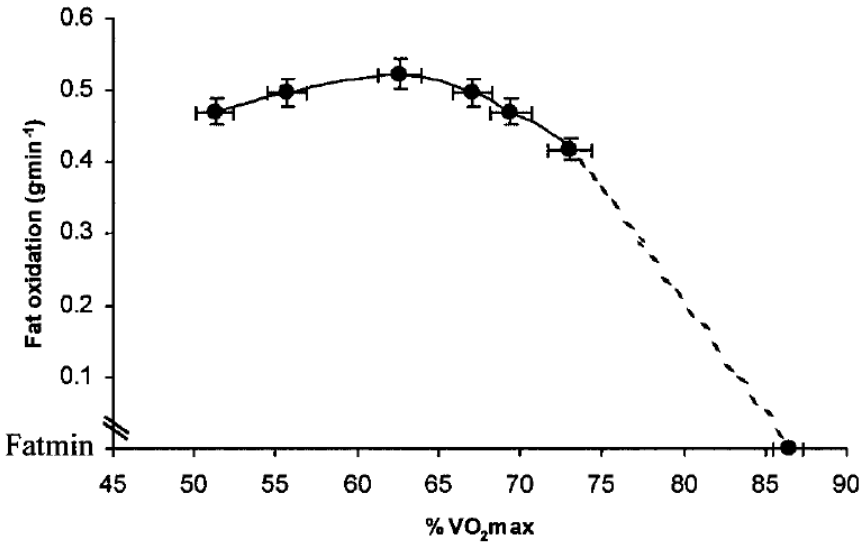

Figure 2-2 The absolute rate of fat oxidation (g/min) as a function of relative exercise intensity (% of $\dot{V}O_2$max). Taken from (Achten and Jeukendrup 2003)

During exercise at a low intensity (Figure 2-3), plasma derived fuels primarily in the form of NEFAs are the preferred substrates for the active muscle. For instance at 25% $\dot{V}O_2$max, Romijn *et al* (Romijn et al. 1993) reported the maximal contribution of plasma NEFA to energy expended as ~80%. Upon the transition to moderate intensity exercise there is an increasing reliance upon substrates stored within the muscle, in particular glycogen but also IMTAG, with the overall contribution of plasma sources remaining fairly constant but with a decline in plasma NEFA contribution and an increase in glucose (Romijn et al. 1993). As described above, the oxidation of all sources of lipids (plasma NEFAs, lipoproteins and IMTAG) is down regulated upon transition from moderate to high intensity exercise, to absolute rates similar or lower than at mild intensities despite the substantially greater energy expenditure (Romijn et al. 1993; van Loon et al. 2001).

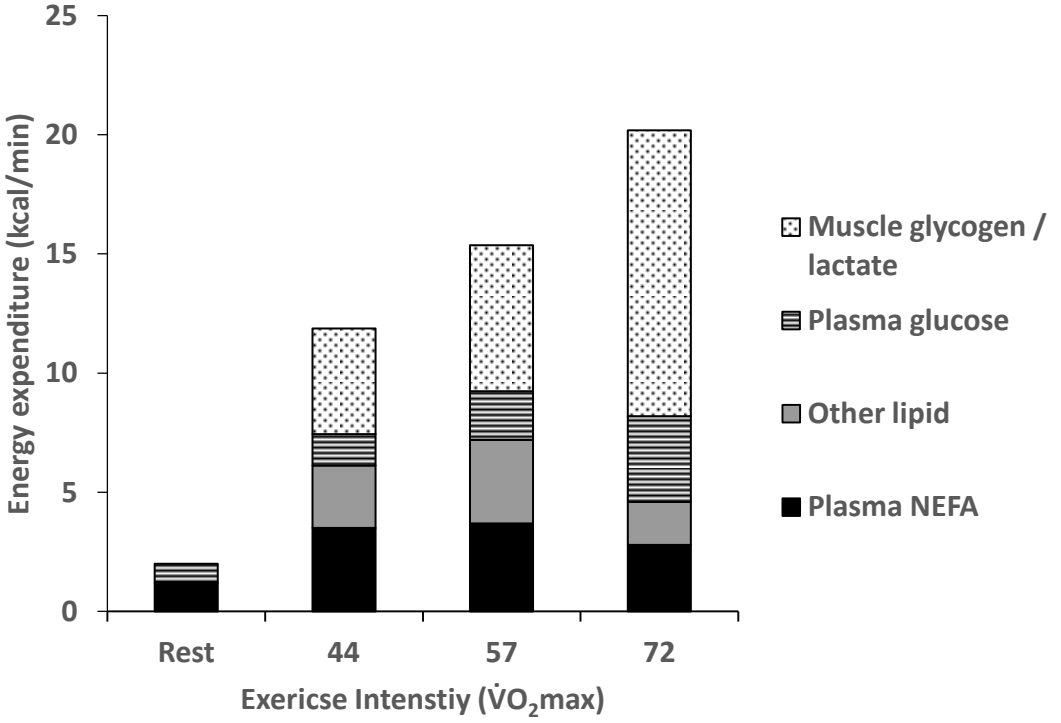

Figure 2-3 The contribution of different substrates to energy expenditure at rest and 3 exercise intensities corresponding to ~44, 57 and 72% $\dot{V}O_2$max. Adapted from (van Loon et al. 2001)

As can be seen in Figure 2-3 there is an ever increasing reliance on carbohydrate (plasma glucose muscle glycogen / lactate oxidation) with increasing exercise intensity, with the contribution of muscle glycogen proceeding almost exponentially with an increase in energy demand, supplying over half (58%) of the requirements at 72% $\dot{V}O_2$max compared to 35% at low-moderate intensities (van Loon et al. 2001).

2.3.2 Duration

It is well established that the duration of moderate to high intensity exercise has a profound effect on substrate oxidation. Even when the intensity of exercise is maintained, there is a time dependent decrease in carbohydrate and increase in fat oxidation (Ahlborg et al. 1974; Romijn et al. 1993). The overall reduction in carbohydrate oxidation as exercise time is prolonged is mostly attributed to the progressive depletion and hence availability of muscle glycogen (Gollnick, Piehl, and Saltin 1974; Watt et al. 2002; Coyle et al. 1986). With the more prominent use of muscle glycogen during the first 120mins of moderate intensity exercise, a maintenance of carbohydrate oxidation is met through an increased reliance on hepatic glucose output (Watt et al. 2002). However, there is also a gradual decrease in the contribution of plasma glucose during very prolonged (>3.5hrs) low to moderate intensity exercise, as a function of decreased plasma glucose delivery as hepatic glycogen stores are gradually depleted (Ahlborg et al. 1974; Ahlborg and Felig 1982; Gonzalez et al. 2015). Under such conditions gluconeogenesis becomes increasingly important for the maintenance of euglycaemia, facilitated by increased circulating gluconeogenic precursors as stated above.

As the duration of moderate intensity exercise is extended, the contribution of fat to energy expenditure is increased, supplied by both IMTAG and the progressively increased rate of appearance (Ra) of plasma NEFA, with this becoming a more important substrate as exercise continues (Watt et al. 2002; Turcotte, Richter, and Kiens 1992; Van Hall et al. 2002). The contribution of IMTAG to energy expenditure appears to be greatest during the initial phase, before the somewhat sluggish NEFA release from adipose tissue lipolysis is adequate to meet the energy requirements (Romijn et al. 1993). When exercise is performed in the

overnight-fasted state the contribution of IMTAG to lipid oxidation is reduced after ~120mins, and plasma NEFA becomes the dominant substrate supplying around 60% of the energy requirements at 57% $\dot{V}O_2$max compared to ~40% over the first 120mins (Watt et al. 2002). The increase in adipose tissue lipolysis is augmented by a gradual rise in catecholamine concentration and a fall in the concentration of the lipolytic inhibitor insulin. The continual rise of plasma NEFA concentration during prolonged moderate intensity exercise implies that the rate of adipose tissue lipolysis is greater than the extraction into the active muscle (Van Hall 2015).

When discussing the impact of exercise duration on substrate oxidation, the interaction with exercise intensity must also be considered. For instance, substrate oxidation and availability in the plasma (NEFA, glucose) at 25% $\dot{V}O_2$max for 120mins of exercise does not appreciably change, with NEFAs providing ~80% of the energy expended. In contrast, during 120mins exercise at 65%$\dot{V}O_2$max there is a progressive increase in the reliance of both plasma NEFA and glucose with no change in carbohydrate or fat oxidation suggesting lesser reliance on muscle glycogen and IMTAG, presumably related to diminished availability (Romijn et al. 1993). At even greater exercise intensities (~82%$\dot{V}O_2$max) when carbohydrate oxidation dominates, small differences in substrate oxidation can still be seen as a function of duration with carbohydrate providing 92%, 89% and 83% of total energy over 60, 90 and 120mins (Torrens et al. 2016).

2.3.3 Training status

It is well-established that endurance based exercise training and the concomitant improvements in aerobic capacity are accompanied by an increase in the reliance of fat and a reduction in carbohydrate during submaximal exercise, with this identified as a component of the cross-over concept (Brooks and Mercier 1994). A greater aerobic capacity, enables a trained individual to perform a given absolute workload at a lower relative exercise intensity (%$\dot{V}O_2$max), with this already discussed as a primary determinant of fuel use, thus enabling greater lipid oxidation. This training induced change has been shown in both cross-sectional studies and longitudinally following endurance training, with greater rates of fat oxidation consistently seen at the same absolute and possibly relative intensity (Hurley et al. 1986; Achten and Jeukendrup 2003; Van Loon et al. 1999; Friedlander, Casazza, Horning, Huie, et al. 1998; Jansson and Kaijser 1987). Although this enhanced ability to utilise lipid is apparent, the importance of carbohydrate at high (>80% $\dot{V}O_2$max) exercise intensities is clear, with it continuing to dominate in both the trained and untrained state (Bergman and Brooks 1999; Friedlander et al. 2006).

Many physiological adaptations to endurance training contribute to the observed increases in fat oxidation. An increased mitochondrial density or mass (Holloszy 1967), along with enzymatic adaptations such as a greater content of citrate synthase and Beta-hydroxyacyl-CoA dehydrogenase (Beta HAD) (Mogensen et al. 2009) ensure the capacity for fat oxidation is enhanced. Furthermore, increased capillarisation of skeletal muscle (Brodal, Ingjer, and Hermansen 1977) and increases in the content or activity of muscle and mitochondrial fatty acid transport proteins (e.g., FABP, FAT/CD36, CPT1 (Talanian et al. 2010; Mole, Oscai, and

Holloszy 1971), results in an increased potential for lipid delivery and uptake by exercising muscle (Kiens et al. 1993) and greater potential for transport across the mitochondrial membrane. Endurance training is also associated with increases in the local stores of fat and/or adaptations to sub-cellular storage locations that would facilitate the capacity for utilisation (Tarnopolsky et al. 2007). In recent years tremendous advances in the understanding of the molecular basis behind endurance training adaptation have been made, and the interested reader is referred to recent review on the topic (Egan and Zierath 2013; Drake, Wilson, and Yan 2016). Collectively, the training adaptations described above lead to a greater cytosolic energy charge (ATP: ADP ratio) and redox status (NADH: NAD^+) resulting in a down regulation of the rate of glycolysis, relieving its inhibitory effect on NEFA uptake and oxidation into the mitochondria (Spriet, Howlett, and Heigenhauser 2000). As well, extra-muscular adaptations such as a greater sensitivity of adipose tissue adrenoreceptors to catecholamine stimulated lipolysis (Mauriege et al. 1997) supports greater systemic NEFA mobilisation and consequent oxidation after exercise training (Friedlander, Casazza, Horning, Buddinger, et al. 1998; Friedlander et al. 1999; Kiens et al. 1993; Turcotte, Richter, and Kiens 1992).

The down regulation of carbohydrate oxidation following endurance training at a given absolute workload results in a reduction in muscle glycogen utilisation (Hurley et al. 1986; Karlsson, Nordesjö, and Saltin 1974) and a reduced plasma glucose uptake and oxidation (Friedlander et al. 1997; Carter, Rennie, and Tarnopolsky 2001; Coggan et al. 1995). The impact of endurance training on glucose flux at the same relative intensity is inconclusive. Due to the increased total energy expenditure and so increased metabolic flux in the trained

state the Rd as a % of total energy from carbohydrate is perhaps the more appropriate expression with some (Carter, Rennie, and Tarnopolsky 2001) but not others (Friedlander, Casazza, Horning, Huie, et al. 1998) reporting a reduction post training. These discrepancies could in part be explained by the relatively small contribution that plasma glucose makes as a proportion of total energy expended during more intense exercise (van Loon et al. 2001; Friedlander, Casazza, Horning, Huie, et al. 1998). A restraint on plasma glucose use irrespective of training status, perhaps reflects the importance of maintaining glycaemia for cerebral metabolism no matter the training status of the muscle (Brooks 2012).

The training induced adaptations described above, particularly those affecting skeletal muscle, are often used to explain greater rates of whole body lipid oxidation when estimated by pulmonary gas exchange. However, greater lipid oxidation in the active muscle following endurance training has not always been seen when investigated more invasively by means of femoral arterial-venous catheterisation with direct blood flow measurements. For instance, after 9 weeks of cycle ergometer training, the leg RQ remained essentially unchanged, from 0.98 at 65% pre training workload, to 0.98 and 1.01 at the same absolute and relative post training intensities (Bergman et al. 1999). However, a similar period of single leg bicycle exercise training did result is a lower RQ in the trained leg compared to the non-trained leg (Henriksson 1977). These discrepancies likely arise through methodological difficulties and limitations in measuring RQ across the working limb. Considering that muscle glycogen utilisation is reduced with exercise training, this is perhaps a truer reflection of events within the muscle.

2.3.4 Diet

Dietary intake, over the short term (i.e. 1-6days) or over a more prolonged period (i.e. >7days) can have profound effects on substrate oxidation during exercise. For instance, the acute effect of carbohydrate ingested in the period immediately before exercise results in an insulin stimulated suppression of whole body lipolysis, a reduction in NEFA oxidation (Horowitz, Mora-Rodriguez et al. 1997) and a decrease in fat oxidation or MFO by ~28% (Achten and Jeukendrup 2003) whilst simultaneously stimulating muscle glucose uptake and oxidation. In contrast, the effects of a single high fat pre-exercise meal are less clear. Although an attenuated raise in insulin and greater elevation in plasma chylomicron TAG and NEFAs compared to a high carbohydrate meal is expected, this does not necessarily translate into an increase in lipid oxidation (Whitley et al. 1998) although others have seen this effect (Rowlands and Hopkins 2002a; Okano et al. 1996). Any effects seen substrate oxidation are likely related to effects on systemic availability of substrates and hormone levels with their direct influence on fuel use.

Although there are many studies that have investigated the effects on metabolism though manipulating the intake or proportion of carbohydrate and fat in the diet, there is lack of consistency as to what qualifies as a high or a low fat/carbohydrate diet. For the purpose of this thesis a high fat diet shall comprise of >60-65% EI from fat (4-5g fat/kg body mass/ day) with low carbohydrate <20% EI / <2 g carbohydrate /kg body mass/ day (Hawley 2011). Either a high fat low carbohydrate (HFLC) or a high carbohydrate low fat (HCLF) diet consumed for 1-3days will substantially impact substrate oxidation during subsequent exercise. The effect of diet over this time period is likely a result of substrate storage and

availability which is a function of the dietary constituents. The pre-exercise myocellular substrate availability is positively correlated with its rate of utilisation (Arkinstall et al. 2004; Shearer et al. 2001; Jansson 1982; Bergstrom et al. 1967; Zderic et al. 2004). This association is evident for both glycogen and IMTAG during moderate intensity exercise (Van Proeyen et al. 2011; Coyle et al. 2001; Zehnder et al. 2006), although, due to its much smaller contribution to total energy expenditure (van Loon et al. 2001), and the dominance of carbohydrate oxidation, the overall impact of IMTAG content is generally less than for glycogen.

A HFLC diet consumed for 5days can increase the rate of fat oxidation during exercise 2 fold compared to a HCLF diet. This substantial change in substrate oxidation following dietary manipulation of this duration, however, is not only an effect of substrate storage, but also the result of cellular adaptations. HFLC diets have been shown to stimulate a greater activity of key enzymes involved in FA catabolism, uptake, and transport such as LPL, HSL, CTP1, Beta HAD, FABp, FAT/CD36, FA translocase (Stellingwerff et al. 2006; Goedecke et al. 1999; Helge and Kiens 1997; Kiens et al. 1987; Cameron-Smith et al. 2003; Yeo et al. 2009), and at least in rodents stimulate mitochondrial biogenesis (Garcia-Roves et al. 2007) together potentially promoting the capacity to oxidise fat. These cellular adaptations accumulate in differences in substrate oxidation at the whole body level with this often referred to as being “fat adapted”.

After a period of “fat adaptation”, rates of whole body lipid oxidation during submaximal exercise remain elevated even after a subsequent 24hr period of high carbohydrate intake that elevates pre-exercise glycogen stores (Burke et al. 2000; Burke et al. 2002; Staudacher et al. 2001; Stellingwerff et al. 2006; Yeo, Lessard, et al. 2008). The greater lipid utilisation following this “fat adaptation + glycogen replenishment” protocol is robust, and persists in spite of additional carbohydrate provision through either a pre exercise carbohydrate meal and/or exogenous carbohydrate ingestion during exercise (Burke et al. 2002). The greater lipid oxidation accumulates in an actual reduction in the reliance of muscle glycogen, with a similar utilisation of plasma glucose (both endogenous (Burke et al. 2000) and exogenous carbohydrate (Carey et al. 2001)). This lower reliance on glycogen however might be attributable to an impairment of the capacity to oxidise carbohydrate, with this same dietary intervention showing a ~ 60% decreased activity of pyruvate dehydrogenase following exercise that involved 60sec sprints – a metabolic stress that should maximally stimulate muscle glycogenolysis, increase PDH flux and the entry of carbohydrate into the TCA cycle for energy production (Stellingwerff et al. 2006). Thus dietary carbohydrate restriction down regulates PDH and carbohydrate metabolism (Raper et al. 2014), which is potentially detrimental to energy production and performance during intense exercise (Stellingwerff et al. 2006).

Thus, it is clear that diet can cause adaptations in substrate oxidation after only a short period, however, it is not clear from the studies highlighted which macro-nutrient is responsible for the induced adaptations. The studies highlighted above compared a high fat low carbohydrate diet to iso-caloric high carbohydrate low fat diet, which by design invariably means altering 2 substrates, it is therefore difficult to localise the source of effect

to a greater provision of fat or a restriction of carbohydrate. Although it was suggested by Bergstrom *et al* (Bergstrom et al. 1967) to be the latter, this has not been comprehensively studied. This concept is covered in more detail later where it became a focus of a dietary intervention in chapter 5.

When the period of exposure to a HFLC diet (combined with endurance training) is prolonged for up to 7 weeks then a similar or greater magnitude of response is seen in whole body substrate oxidation. Compared to a HCLF diet consumed over the same period, those on a HFLC diet had a lower RER (0.86 vs 0.93), leg RQ (0.87 vs 0.91) with a greater uptake and oxidation of plasma NEFA and VLDL-TAG, and an apparent sparing of the lower starting muscle glycogen content (Helge, Richter, and Kiens 1996; Helge, Wulff, and Kiens 1998; Helge et al. 2001). However, after a 1 week period of carbohydrate restoration, the effects of the HFLC diet had largely dissipated and were similar between the conditions. Exposure to a more extreme and divergent dietary intake of fat and carbohydrate, with carbohydrate restricted to <20g a day for 28days, and is designed to induce a ketogenic state, can result in an even greater reliance on lipid during exercise. In 5 trained cyclists, this dietary approach lowered the RER during exercise at ~63% $\dot{V}O_2$max from 0.83 on the HC to 0.72 after the ketogenic HFLC diet. This was accompanied by a lesser reliance on muscle glycogen and plasma glucose during the exercise bout although the muscle glycogen content was ~50% lower pre-exercise than the HC comparison.

Although the studies described above report profound effects on substrate oxidation by manipulation of the total dietary content of fat or carbohydrate, over the last decade or so evidence has accumulated to suggest that the timing of when these macronutrients are consumed in relation to training may also be as important. As described in section 2.3.1, exercise training results in an alteration to the muscle phenotype, improving fatigue resistance and the capacity to oxidise fat. The molecular pathways activated by exercise that are thought to be responsible for these adaptive responses (Egan and Zierath 2013) share similar pathways and interact with nutrient sensing proteins such as AMPK (adenosine monophosphate-activated protein kinase), p38MAPK (P38 mitogen-activated protein kinases) or SIRT1 (sirtuin (silent mating type information regulation 2 homolog) (Hardie 2011). These signalling proteins are thought to stimulate the transcriptional co-activator PGC1 alpha (Peroxisome proliferator-activated receptor gamma coactivator 1-alpha) (Jäger et al. 2007) ultimately leading to an increase in mitochondrial biogenesis. The provision of carbohydrate prior to, during or post exercise can directly down-regulate the activation of AMPK and expression of PGC1alpha (Philp et al. 2013; McBride et al. 2009; Psilander et al. 2012) and so with-holding or restricting carbohydrate intake in and around training increases AMPK activity ultimately augmenting mitochondrial enzyme activity and content (Morton et al. 2009; Bartlett et al. 2013; Van Proeyen et al. 2010) and whole body lipid oxidation (Yeo, Paton, et al. 2008). For a recent review on the molecular mechanisms underpinning endurance training and nutrient interactions, the interested reader is referred to a recent review (Close et al. 2016). With the molecular adaptations to endurance training evidently augmented with a restriction in carbohydrate availability, several “train-low”/ “sleep-low”

(regarding low carbohydrate availability) type strategies have emerged as ways to enhance whole body lipid oxidation (Marquet et al. 2016).

To summarise, the dietary intake of both carbohydrate and fat alter the availability of substrates and hormones within the circulation that either directly or indirectly alter substrate oxidation within the muscle. Dietary macronutrient composition (in particular carbohydrate intake), also substantially impacts myocellular substrate storage, which effects substrate selection during exercise and can modulate the magnitude of training induced adaptations favouring fat oxidation. The diet is therefore a powerful and easily manipulated regulator of exercise substrate metabolism.

2.3.5 Sex

Investigating sex based differences in substrate metabolism is difficult and problematic due to the large number of potentially confounding variables with adjustments often needed for body composition or rigorous matching of subjects (Tarnopolsky 2008). Nonetheless, when groups have been well matched for factors such as aerobic capacity the most consistently reported sex related difference in exercise metabolism, is that women, compared to men, rely more heavily on lipid and less on carbohydrate oxidation during exercise at the same relative submaximal intensity (reviewed in (Tarnopolsky et al. 1990; Lundsgaard and Kiens 2014; Tarnopolsky 2008). Using pulmonary indirect calorimetry, although only modestly greater than men, women demonstrate significantly greater MFO (7.4 ± 0.2 vs. 8.3 ± 0.2 mg/kg FFM/min) (Venables, Achten, and Jeukendrup 2005) generating a greater proportion

of energy through fat oxidation across a range of exercise intensities (35-85% $\dot{V}O_2$max) (Cheneviere et al. 2011). Yet when more invasive measures have been taken to determine the source of the different substrates oxidised, the findings have been less conclusive (Tarnopolsky 2008).

Regarding carbohydrate metabolism, women have a lower rate of appearance (Ra), rate of disappearance (Rd) and metabolic clearance of glucose than men, indicating that they have a lower whole-body reliance on circulating blood glucose during moderate intensity exercise (Friedlander, Casazza, Horning, Huie, et al. 1998; Carter, Rennie, and Tarnopolsky 2001; Devries et al. 2006). It is not known which process is down regulated in women - hepatic gluconeogenesis, glycogenolysis or both. Despite apparent whole-body differences in glucose turnover, the contribution of blood glucose to substrate oxidation across an exercising limb estimated using a-v balance methods has been observed to be similar in men and women (Roepstorff et al. 2002). The effect of sex on muscle glycogen utilisation is inconsistent. Some have observed similar rates of muscle glycogen utilisation during prolonged cycling exercise (Tarnopolsky et al. 1995; Roepstorff et al. 2002; Zehnder et al. 2005), whereas Tarnopolsky *et al* (Tarnopolsky et al. 1990) reported ~25% less muscle glycogen use in women as compared to men during a 90-101mins treadmill run at 65%$\dot{V}O_2$max, and a fibre specific 50% less use following high intensity bike sprinting exercise (Esbjörnsson-Liljedahl et al. 1999). Some of the discrepancies in the literature could be accounted for by differences in the mode of exercise (e.g., cycling vs. running) or other aspects of methodology such as measurement technique (e.g., mixed vs. fibre specific glycogen use, determination of total, pro- or macro-glycogen) or control for menstrual cycle

phase (Devries et al. 2006; Esbjörnsson-Liljedahl et al. 1999). Thus although the whole-body rate of carbohydrate oxidation is clearly lower in women, identifying which source of carbohydrate is spared has been difficult to isolate.

The consistently reported greater whole body lipid utilisation in women compared to men appears to come from multiple sources. Determined by the Ra of glycerol expressed relative to BM, women have a greater whole body lipolytic response during exercise than men (Carter, Rennie, and Tarnopolsky 2001; Davis et al. 2000; Friedlander, Casazza, Horning, Huie, et al. 1998) resulting in greater NEFA availability, uptake and oxidation (Roepstorff et al. 2002; Mittendorfer, Horowitz, and Klein 2002; Burguera et al. 2000; Romijn et al. 2000; Wallis et al. 2006; Friedlander, Casazza, Horning, Buddinger, et al. 1998; Friedlander et al. 1999). Following endurance training, women also show a 2 fold increase in the rate of NEFA oxidation, whereas there is a reduction in NEFA flux and oxidation seen in men post-training compared to the same relative pre-training intensity (Friedlander, Casazza, Horning, Buddinger, et al. 1998).

The influence of sex on IMTAG use is less conclusive. Although a greater IMTAG utilisation during exercise in women has been reported (Roepstorff et al. 2002; Steffensen et al. 2002), this is not a universal finding (Devries et al. 2007; White et al. 2003; Zehnder et al. 2005). However, it must be recognised that the quantification of IMTAG by commonly used techniques such as mixed muscle biochemical analysis or MRI can be insensitive, which may limit interpretation of the existing literature (Watt, Heigenhauser, and Spriet 2002).

Nonetheless, women do appear to have greater IMTG storage than men, along with a higher number of small IMTAG droplet and proportion of IMTAG/mitochondria interactions post exercise indicating a greater propensity for IMTAG utilisation (Devries et al. 2007) or it is an artefact of the well-recognised finding that women have greater resting IMTAG content which determines the rate of use (Steffensen et al. 2002; Roepstorff et al. 2002; Devries et al. 2007; Tarnopolsky et al. 2007; Høeg et al. 2009).

As discussed later in section 3.1 the oxidation of protein or BCAA during exercise is quantitatively much smaller than that of carbohydrate and fat, and so is often not determined or considered when making inferences about substrate oxidation. Despite this, women have been shown to have lower rates of leucine oxidation (representing BCAA oxidation) both at rest and during moderate intensity exercise (Phillips et al. 1993), with these differences persisting following endurance training (McKenzie et al. 2000). Therefore, although protein oxidation is generally not accounted for in sex based comparisons, and its overall contribution is only small, it could mask or obscure potentially small sex based differences in substrate metabolism having the effect of lessening the calculated fat oxidation.

2.3.5.1 Factors responsible for sex differences in substrate metabolism

2.3.5.1.1 Hormonal mileu

After puberty the hormonal environment between men and women becomes markedly different, with this change responsible for many of the typical sex based differences in size,

adiposity, body composition, and substrate oxidation (Comitato et al. 2015). Prior to the menopause, the circulating concentration of oestrogen, even during the early follicular phase of the menstrual cycle when it is at its lowest, is still ~2 fold higher in women than men (Stachenfeld and Taylor 2014). With oestrogen receptors found ubiquitously throughout the body including skeletal muscle (even in men) then there is potential for it to be a driving factor behind difference in metabolism during exercise (Oosthuyse and Bosch 2012). Circulating progesterone levels are also substantially different between men and women; however, as described in more detail in section 4.2, the independent role of progesterone on substrate metabolism has not been adequately assessed.

Perhaps the most convincing evidence that oestrogen is a major regulator of substrate oxidation is the effect it has when administered to men or amenorrhoeic women. For instance, 8 days administration in men results in a ~30% increase on fat oxidation and ~10% decrease in carbohydrate oxidation during exercise at 65% $\dot{V}O_2$max (Hamadeh, Devries, and Tarnopolsky 2005). This occurs alongside a reduction in hepatic glucose output (Ra) and (Rd) indicative of reduced flux, but does not appear to change muscle glycogen utilisation (Carter et al. 2001; Devries et al. 2005). Oestrogen administered for a few days also increases plasma NEFA concentration during exercise in either amenorrhoeic women or men although it does not seem to alter whole body lipolysis with similar glycerol Ra and Rd (Ruby et al. 1997; Carter et al. 2001). Nonetheless, the directional consistency of the findings mentioned for oestrogen to favour lipid oxidation whilst reducing hepatic carbohydrate reliance is suggestive of oestrogen's important role in the sex based differences seen in substrate oxidation.

Despite greater insulin induced glucose uptake in women than men (Høeg et al. 2009) in part attributed to oestrogen, oestrogen supplementation in men appears to have little effect on carbohydrate metabolism. Whilst oestrogen supplementation for 8 days increased mRNA abundance of GLUT4 (3.9 fold) and glycogen synthase (GS) (1.3 fold) there were no changes in abundance of glycogen phosphorylase, hexokinase II, Phosphofructokinase (Fu et al. 2009) with similar GLUT4 skeletal muscle protein content also shown when sampling from men and women matched for $\dot{V}O_2max$ (mg/kg FFM/min) (Høeg et al. 2009). Although small differences might indicate the potential for greater glycogen storage following oestrogen supplementation, this has not been reported when directly measured, and is consistent with no change in muscle glycogen breakdown during exercise following oestrogen supplementation (Carter et al. 2001; Devries et al. 2005).

In addition to the substantial sex based differences in oestrogen concentration, the circulating concentration of testosterone is more than 10-fold higher in men, which could reasonably contribute to the differences in metabolism, although this is not thought to be the case. Pharmacologically inducing high (10ng/ml) or low (0.5ng/ml) testosterone in healthy men resulted in no difference in whole body substrate oxidation rates, liver glucose release or estimated muscle glycogen utilization over 90mins endurance exercise (Braun et al. 2005).

2.3.5.1.2 Catecholamines

Differences in the autonomic nervous system response to exercise are thought to contribute to the sex based differences in circulatory substrate availability and oxidation. During endurance exercise women demonstrate lower sympathetic nerve activity than men (Jones et al. 1996), with lower circulating epinephrine and norepinephrine thereby attenuating the stimulatory effect of epinephrine on rates of muscle glycogenolysis and carbohydrate oxidation (Horton et al. 1998). Whilst the lower circulating epinephrine would be expected to result in a lower whole body lipolytic response (Mora-Rodriguez et al. 2001), it appears that women are more sensitive to the lipolytic actions of epinephrine with greater rates of lipolysis for the same amount of infused epinephrine, accumulating in greater circulatory glycerol and NEFA (Horton et al. 2009; Schmidt et al. 2014). The difference in lipolytic response between men and women is a result of differences in the stimulation of the different adrenergic receptors. In women, mainly the lipolytic Beta-adrenoreceptors are activated (Arner et al. 1990), whereas these are activated in men alongside antilipolytic alpha-adrenoreceptors (Wahrenberg, Bolinder, and Arner 1991; Wahrenberg, Lönnqvist, and Arner 1989; Schmidt et al. 2014), achieving a greater overall net lipolytic response in women.

2.3.5.1.3 Muscle morphology and metabolic characterstics

Although large inter-individual variation in fibre type distribution exists, it is generally reported that women have a greater number of type 1 muscle fibres and when expressed relative to area, the proportion of type 1 fibres are ~30% greater in women, with the proportion of type 2a or 2x greater in men (Steffensen et al. 2002). The greater preponderance of the more oxidative type 1 fibres in women along with a greater ratio of

the glycolytic type 2 to 1 fibres in men (Simoneau and Bouchard 1989; Høeg et al. 2009; Miller et al. 1993) is supportive of the differences in RER often reported. Although there are no sex based differences in the number of capillaries surrounding each muscle fibre, due to the lower total amount of type 2 fibres and smaller individual area of these fibres, women have a greater capillary density per muscle area, with a theoretical shorter capillary to fibre diffusion distance (Høeg et al. 2009; Roepstorff et al. 2006). These differences could enable a better perfusion of substrates and oxygen to a larger relative area of oxidative fibres, accumulating in less relative metabolic stress and better maintenance of cellular energy balance. This is reflected by a lesser increase in free AMP, the ratio of free AMP/ATP and AMPK phosphorylation during 90mins cycling at 60% $\dot{V}O_2$max in women than well matched men (Roepstorff et al. 2006).

In line with the often reported observation of greater IMTAG storage in women than men, women display increased expression of genes linked to fat storage, specifically Sterol regulatory element-binding protein 1 (SREBP1c) and mitochondrial glycerol phosphate acyl transferase (mtGPAT), of which oestrogen supplementation in men increases the mRNA content of (SREP-1c (3 fold) and mtGPAT (1.4 fold)) (Fu et al. 2009). Moreover, women express a greater mRNA content of fatty acid transport protein (FATP) and cytosolic fatty acid binding protein (FABPc) and CPT1 (Tarnopolsky 2008) along with greater protein expression of FAT/CD36 irrespective of training status (92). Thus the capacity to transport lipid across the plasma membrane is greater in women which could augment lipid oxidation.

As well, the glycolytic capacity of muscle however does appear to be greater in men than women, with muscle homogenates of untrained men showing greater activity of glycogen phosphorylase, pyruvate kinase, PFK and lactate dehydrogenase than untrained women (Green, Fraser, and Ranney 1984). Although this could be an artefact of the greater proportion of type 2 fibre types in men, it is still suggestive of a greater capacity for glycolysis and glycolytic flux in men. The greater ratio of the activity of 3-hydroxyacyl CoA dehydrogenase (HAD) to glycolytic enzymes in women indicates that women have a lower glycolytic potential relative to the potential for beta-oxidation (Green, Fraser, and Ranney 1984)

In summary, the fundamental differences in gene expression or molecular protein levels of key FA transporters, or genes involved with lipid synthesis, indicate that women are inherently better suited for fatty acid transport and storage in and around muscle. The additional greater potential for Beta oxidation and IMTAG turnover than men, along with the morphological differences in muscle fibre type composition and capilarisation, are likely to be collectively responsible for the preponderance of greater fat oxidation in women than men during exercise.

2.4 Variability in substrate oxidation

2.4.1 Variability of fuel use

It was demonstrated many years ago through the measurement of RER that the contribution of carbohydrate and lipid to energy production varies during exercise as a function of both

intensity and duration (Christensen and Hansen 1939). Since these early observations several key factors have been identified that can influence substrate utilisation during exercise (see section 2.3 for further details). However, even when many of these factors are accounted for, there is still a large degree of inter-individual variation in the proportion of fat and carbohydrate oxidised during exercise. For instance, Helge and colleagues reported the RER to range between 0.83 and 0.95 during 60mins of bicycle exercise at 57% $\dot{V}O_2$max, despite controlling for sex (studying men only), training status and dietary intake for the 3-days prior to testing (Helge et al. 1999).

A similar degree of variability in RER was also reported in a fairly homogenous cohort (*n* 61) of well-trained cyclists when exercising at 41, 63, and 80% $\dot{V}O_2$max, with the degree of variability in resting RER (range 0.718-0.927), stable across the exercise intensities reported (Goedecke et al. 2000). Goedecke *et al* were able to explain between 42-56% of the variability in RER at the different exercise intensities. The variables that most consistently predicted RER across the 3 different exercise intensities included exercise training history (METS/wk), dietary fat intake (%EI), skeletal muscle CS activity and plasma NEFA concentration which were all negatively associated with RER (i.e., greater fat oxidation). The only consistent positive predictor of RER (i.e., greater carbohydrate oxidation) was pre-exercise muscle glycogen content. Notwithstanding the useful insights gained from the invasive measures taken by Goedecke *et al*, the relatively small and homogenous group of well-trained athletes measured potentially limits the applicability of the findings to normally active individuals. Additionally, although able to attribute variance in RER to dietary fat intake (% EI), with only 63 participants the study was unlikely to be adequately powered to

detect other subtle differences in dietary intake that might have meaningful effects on the variability of substrate oxidation.

In regards to some of these pitfalls, the work of Venables *et al* (Venables, Achten, and Jeukendrup 2005) is particularly relevant. From a much larger (*n* 300) and broader scope of men and women ([age: 18-65], of varying body composition [6-41% body fat] and aerobic fitness [$\dot{V}O_2$max: 21-83ml/kg/min]), an uphill walking/running treadmill based "FatMax" test was employed to quantify MFO over a wide range of exercise intensities. A large 5-fold inter-individual variability in MFO was seen, with this attributed to $\dot{V}O_2$max, SRPAL, sex and body composition. However, the analysis still left 65% of the variability unexplained, with the authors concluding that variables not accounted for such as dietary intake likely explaining a sizable part of this. This suggestion is supported by the consistent negative influence of dietary fat intake (% EI) on the variability of RER reported by Goedecke *et al* (Goedecke et al. 2000) and the robust impact that dietary manipulation has on substrate oxidation (Helge, Wulff, and Kiens 1998; Bergstrom et al. 1967; Phinney et al. 1983), thus, their conclusion seems justified and worthy of further investigation.

Moreover, although the work of Venables *et al* offers insights and helps explain a sizeable proportion of the intra-individual variability in substrate oxidation, methodological limitations and statistical caveats perhaps conceal or preclude a true assessment. Firstly, the participants were not familiarised to the testing procedure prior to the measurements of substrate oxidation which due to the novelty of the task and potential for a catecholamine induced stress response could have impacted the fuels used over the exercise bout. Secondly, measurements of MFO were made after only a minimum of a 4hr fast, with the

effects of a pre-exercise meal still evident for 6hrs (Montain et al. 1991) this lack of standardisation may also have perturbed a true measurement of MFO. In addition, the fairly crude measurement of SRPAL by questionnaire and the estimation of body composition by skinfold measurement may also have impacted the findings. In spite of the fact that the men were heavier, taller, with more FFM, were more physically active and aerobically fitter ($\dot{V}O_2max$) than the women who had a higher body fat%, no statistical accounts were made for these differences when explaining the variability seen in substrate oxidation. Thus although Venables *et al* reported sex to be a significant determinant of the variability in MFO, this was likely confounded by the differences in body size / composition and aerobic capacity that were not appropriately accounted for and so this area became an area of focus in Chapter 4.

2.5 Sex specific nutritional aspects

The numerous sex-based differences in physiology and metabolism (described in more detail in 2.3.5) accumulate in women generating a greater proportion of energy from lipid oxidation than men over a large range of exercise intensities (Cheneviere et al. 2011; Venables, Achten, and Jeukendrup 2005). Additionally, dietary intake has a substantial impact on substrate utilisation during exercise, for example, a HFLC adaptation diet in men results in greater lipid oxidation and less reliance on muscle glycogen even after carbohydrate restoration (Burke et al. 2002). The greater preponderance for fat oxidation during exercise suggests that women may have a greater capacity to adapt to a HFLC adaptation diet to further augment lipid oxidation. Despite substantial changes in substrate

oxidation seen in men following a HFLC diet these do not often translate into improvement in exercise performance (Burke 2015). Women on the other hand, have a greater maximal rate of fat oxidation than men, that also occurs at a higher exercise intensity (Venables, Achten, and Jeukendrup 2005) and so might be able to capitalize on this and improve endurance performance after a HFLC adaption diet.

2.5.1 Sex differences in response to dietary intake

As described in section (2.3.4) the availability of substrates in and around the muscle is heavily influenced by diet and is one of the key determinants of substrate oxidation during exercise. Women possess a ~30% greater insulin stimulated glucose uptake over men, with a 56% greater HKII protein content facilitating continual glucose uptake (Høeg et al. 2009; Hoeg et al. 2011) which when combined with similar glycogen synthase mRNA (Fu et al. 2009) suggests a greater capability to store carbohydrate in women. On the contrary, it was originally proposed that when women followed the typical carbohydrate ingestion guidelines (75% EI carbohydrate (Costill 1988)) to achieve glycogen super compensation, they were unable to achieve similar levels as men (Tarnopolsky et al. 1995). However, as acknowledged by the authors, this was a reflection of the lower total energy intake and therefore an insufficient total amount of carbohydrate ingested by the women (<8g carbohydrate kg FFM/day). It was later shown that increases in carbohydrate intakes to 10-12g/kg FFM is sufficient to achieve glycogen super-compensation in men and women (Tarnopolsky et al. 2001; James et al. 2001).

The carbohydrate induced anti-lipolytic actions of insulin are well known, with insulin inhibiting ATGL and HSL in adipose tissue to decrease circulating NEFA, NEFA uptake and lipid oxidation (Coyle et al. 1997). Exogenous carbohydrate administration during exercise results in a similar insulin and metabolic response between the sexes, with similar Ra of glucose and contribution of the exogenous carbohydrate to total energy expenditure (Tremblay et al. 2010; Wallis et al. 2006). This comparable response to carbohydrate administration between the sexes, also leads to a similar estimated minimal rates of muscle glycogen utilisation and rates of whole body lipid oxidation, with the exogenous carbohydrate almost normalising the whole body substrate oxidation in women to that of men (Wallis et al. 2006; Riddell et al. 2003).

Despite a similar capacity for glycogen storage between men and women, it could be argued that the necessity to glycogen super-compensate for endurance exercise performance may be somewhat attenuated in women. The greater propensity for lipid oxidation during exercise in women, with the hormonal, physiological and molecular machinery to capitalise on the greater availability of IMTAG and plasma NEFAs potentially lessens the importance of glycogen as an energy source.

As described in section 2.3.4, studies in men demonstrate that a HFLC adaptation diet consumed for >5days enhances the capacity to oxidise lipid during moderate intensity exercise, with these adaptations persisting even after carbohydrate restoration (Yeo et al. 2011). However, most of the data pertaining to HFLC induced alterations were carried out in

men and with limited well controlled interventions in women. It is not clear if women possess the same scope as men for dietary induced fat adaptations or if women are already achieving near maximal rates. The few investigations of dietary fat/carbohydrate manipulation in women although of generally poor design are described below.

Over a period of 7days, women cyclists self-selected a diet intended to be low (13% EI), moderate (54% EI) or high (72% EI) in carbohydrate content (O'Keeffe et al. 1989). The RER recorded during cycling exercise at 80% $\dot{V}O_2$max was assessed at 15, 45, 90, and 120mins of the exercise bout. On average the RER was highest following the high carbohydrate diet compared to both the moderate and low diets, with no differences between the low and moderate. However, the "self-selected" nature of these trials also led to differences across all 3 macronutrients, with the fat and protein content (grams and % total EI) also decreasing significantly from low to moderate to high carbohydrate intakes, and so making meaningful inferences about the cause of the difference in RER difficult. It is also somewhat surprising that the relatively small ~ 16% difference in EI from carbohydrate in the high carbohydrate trial compared to the moderate trial induced a difference in RER that was not evident in the more extreme ~ 41% difference between the low carbohydrate and moderate trial. The greater difference in total carbohydrate intake and greater carbohydrate restriction would be anticipated to see the largest effect.

Using a self-selected dietary intervention, men and women runners were asked to consume over a period of 4wks a diet that was either low (16%), moderate (31-33%) or high (44%) in

fat (Horvath et al. 2000). In the male runners, no differences in RER were reported between the different diets determined 3hrs post-prandially whilst running at 80% $\dot{V}O_2$max to exhaustion. However, somewhat counter intuitively, in the women, the RER following the moderate fat diet (0.96±0.01) was significantly higher than after consuming the low fat diet (0.94±0.01) indicating greater fat utilisation with greater carbohydrate intake. It is difficult to interpret the findings from this study, with the authors acknowledging that the "runners did not follow the diets accurately…..with half of the subjects in the high fat group not actually increasing fat intakes above the moderate intake period". Additionally, to highlight the poor dietary control, the runners were unintentionally in severe (~1000kcal/day) negative energy balance with this likely impacting exercise substrate oxidation (Braun and Brooks 2008). The substrate oxidation data were also collected at 80% $\dot{V}O_2$max, with lactate levels between 11 and 16mmol/L it was therefore likely that the substantial metabolic acidosis and buffering of CO_2 invalidates any estimates of substrate oxidation.

Whereas the work of O'Keefe *et al* (O'Keeffe et al. 1989) did not observe a difference in RER between the 13% and 54% carbohydrate diets (59% and 25% fat, respectively), this was not the case in a more rigorously controlled intervention. When trained men and women were provided with diets containing either moderate fat (35% EI) or low fat (15% EI) for 3 days, a higher RER over a 20min of run at ~73% $\dot{V}O_2$max was reported for the low compared to the moderate fat trials (0.95±0.01 *vs* 0.93±0.01, respectively) (Larson-Meyer et al. 2008). Following a 24hr high carbohydrate glycogen replenishment period, no differences in RER were then seen over a 90min run at 63% $\dot{V}O_2$max conducted 3hrs post-prandially. Unfortunately, the data in the aforementioned study are not provided separately for men

and women but the authors declared there to be no sex based differences in RER. This data does however support the assertion that a period longer than 3days of dietary carbohydrate restriction / high fat intake is required to induce true metabolic and cellular adaptations promoting lipid oxidation, although the small range of dietary fat intakes provided precludes this assertion for truly high fat intakes. Overall, the direction of change in substrate oxidation in women following high fat or low carbohydrate diets appears to be similar to men. However, there is a lack of well controlled dietary interventions conducted over sufficient duration to fully capture the magnitude of change and if it impacts endurance performance, and so this became a focus of Chapter 5.

2.6 Summary and thesis aims

A large proportion of the substantial inter-individual variability in the capacity to oxidise lipid during exercise is un-accounted for (Venables, Achten, and Jeukendrup 2005). It is clear that habitual dietary intake has the potential to profoundly impact substrate oxidation during exercise directly through mediating substrate storage (Bergstrom et al. 1967; Zderic et al. 2004), or indirectly through modulating training induced adaptations that impact substrate oxidation (Hawley 2011). It is therefore likely that habitual dietary intake explains a degree of the variability seen in lipid oxidation whilst physically active although this has yet to be quantified.

Moreover, consumption of a high fat diet has the potential to enhance lipid oxidation during exercise and potentially spare the limited glycogen stores (Burke et al. 2002; Carey et al.

2001). The majority of exercise metabolism research has been conducted in men, with the effects of a high or low fat dietary intake no exception. The few high fat dietary intervention studies that have been attempted in women have either been poorly controlled or adhered to (O'Keeffe et al. 1989; Patterson and Potteiger 2011) or only short duration and of only moderate fat intake (Larson-Meyer et al. 2008). There are numerous metabolic differences known to exist between men and women and in particular the propensity for women to oxidise more lipid than men during exercise. It is not known however if women respond in similar manner to men to dietary fat manipulation and increase the already elevated rates further, with the potential for this to impact exercise performance also unknown.

A common virtue of well controlled iso-caloric dietary manipulation studies involves the substitution of one macronutrient with that of another. Any observed effect of this manipulation however, could be a result of either the restriction or the replacement of the respective macronutrient, therefore attributing causality is difficult. To this extent, hyper-caloric dietary interventions make it possible to manipulate the intake of just a single macronutrient and thus enable a better detection of cause. When this model of dietary manipulation (additional lipid provision) has previously been employed, there were inconsequential differences in substrate oxidation, however, it is quite likely the dietary intervention period (1.5 d) was not of sufficient duration to elicit a change (Decombaz et al. 2013; Zehnder et al. 2006).

Thus the primary aims of this PhD were:

1. To better understand the determinants of the variability in substrate oxidation during exercise, with particular reference to the influence of nutrition.
2. To comprehensively characterise the metabolic response in women during moderate-hard intensity exercise following diets high in fat but limited in carbohydrate or high in fat and not limited in carbohydrate. With a subsidiary aim of characterising this response in users of a progesterone only form of hormonal contraceptive.
3. To investigate the impact of this dietary manipulation on endurance exercise performance, and markers of health in well-trained women.

3 GENERAL METHODS

Within this chapter a description of the methods applied throughout this thesis is provided, detailing the key principles of specific methods along with justification for their use and limitations of the approaches.

3.1 Calculation of substrate oxidation: principles, assumptions and measurement techniques

3.1.1 Principles and stoichometry

Directly measuring energy expenditure requires measuring the heat generated by the body dissipated into the environment, with this heat representing the combustion of macronutrients (Schutz 1995). This direct measure of calorimetry is considered the gold standard method to measure energy expenditure, however due to costs, extreme subject burden and the confinement to an insulated room calorimeter the methodology is not widely used. The more commonly used approach to determine energy expenditure is through indirect calorimetry. With this approach, pulmonary gas exchange measurements of oxygen ($\dot{V}O_2$) and carbon dioxide ($\dot{V}CO_2$) are used to estimate the rate and type of substrate oxidised (Frayn 1983) and by inference total energy. Indirect calorimetry has been shown to correlate highly to direct calorimetry (Seale et al. 1990) with the advantage of the former being much more practical.

The different chemical composition of fats, carbohydrates and proteins, require varying amounts of O_2 during the combustion of each substrate, producing varying amounts of CO_2 in the process. By measuring the ratio of $\dot{V}CO_2$ to $\dot{V}O_2$ it is therefore possible to estimate the relative mixture of fuels being oxidised at either rest or exercise. For example, if carbohydrate (e.g., glucose) is the only fuel of use then the RER is 1.0, with the stoichiometry:

$C_6H_{12}O_6 + 6O_2 = 6CO_2 + 6H_2O$

RER = 6/6 = 1.0

The stoichiometry can vary slightly depending upon the carbohydrate used in the calculation although the impact on the RER is small, especially in comparison to that of the different lipids and amino acids (Frayn 1983).

Using the most common lipid of Trioleate ($C_{57}H_{104}O_6$) as an example, the stoichiometry and RER for this FA is:

$C_{57}H_{104}O_6 + 80O_2 = 57CO_2 + 52H_2O$

RER = 57/80 = 0.71

The RER of triglycerides vary between 0.69 and 0.76 with differences in the RER a result of the large differences in fatty acid chain length (Livesey and Elia 1988).

Calculating the contribution of protein oxidation to the RER is somewhat more complicated. Protein must first be broken down into amino acids (AAs) and either transaminated or deaminated before it can be oxidised, with the resultant nitrogen or sulphur being excreted

in urine, faeces and sweat (Brooks 2012). The remaining keto-acid is oxidised in a similar manner to fat and carbohydrate, with the stoichiometry and RER for plasma albumin as a representative protein equating to:

$C_{72}H_{112}N_2O_{22}S + 77\ O_2 = 63\ CO_2 + 38\ H_2O + SO_3 + 9\ CO(NH_2)_2$

RER = 63/77 = 0.82

The RER of protein varies depending on the amino acid oxidised, with 0.85 being the typical value employed.

Substrate oxidation rates in this thesis were calculated using the equations of Frayn (Frayn 1983) without correcting for protein oxidation under the assumption that this would be negligible (see section 2.2)

Rate of carbohydrate oxidation (g/min) = $(4.55 * \dot{V}CO_2[L/min]) - (3.21 * \dot{V}O_2[L/min])$

Rate of fat oxidation (g/min) = $(1.67 * \dot{V}O_2[L/min]) - (1.67 * \dot{V}CO_2[L/min])$

There are several different equations available to calculate substrate oxidation rates from $\dot{V}O_2$and $\dot{V}CO_2$ measurements, each differing slightly due to small differences in stoichiometry with the different type of carbohydrate, amino acid or fatty acid used in the derivation of the equation. The use of different FAs in the different equations can be justified with many different FAs oxidised during exercise (Krishnan and Cooper 2014), with a rationale given for the FA chosen with each equation. For instance, Frayn (Frayn 1983) justified using palmytoyl-stearoyl-oleoyl glycerol as this triacylglycerol is very similar to the average composition of

human adipose tissue which under resting conditions is where the bulk of FAs oxidized are derived from. In contrast, Consolazio (Consolazio 1963) based equations on palmitic acid as this is the most abundant FA in the average human diet and plasma. As summarised by Jeukendrup & Wallis (Jeukendrup and Wallis 2005), the different substrates used in the equations with differences in stoichiometry can result in variation in calculated oxidation rates of carbohydrate and lipid oxidation during exercise (~6% and ~3%, respectively).

The equations of Frayn (Frayn 1983) for carbohydrate oxidation is based on an exclusive oxidation of glucose, this disregards the potentially important contribution of muscle glycogen oxidation during exercise. Slightly different stoichiometry arises if glycogen is used instead of glucose with calculated rates of carbohydrate oxidation ~10% (Jeukendrup and Wallis 2005). However, without a direct measure of muscle glycogen utilisation potential errors of unknown magnitude could be made therefore the use of glucose as the representative carbohydrate was judged to be more appropriate and also enable better comparisons to be made with the bulk of previous literature in this area.

3.1.2 Assumptions

The measurement of whole body (pulmonary) gas exchange is often used to reach conclusions about the energetics of working muscle. This assumes that the working muscles dominate the whole body response, and the whole body response reflects that occurring in the working muscle. Measurements at the working muscle are rarely made due to the invasiveness and difficulties in measuring blood flow, O_2 and CO_2 contents of arterial /

venous blood, with further confounding of the catheter position for sampling (retrograde or antegrade) (Van Hall et al. 2002), which influences the mixing of blood from other non-muscular or auxiliary muscle sites. Nevertheless, although certain assumptions are made through the measurement of whole body breath measurements, when they have been combined with invasive measures of A-V differences similar findings have been reported, with small (0.03) greater RQ at the muscle than whole-body (Friedlander et al. 2006; Bergman et al. 1999; Helge et al. 2001). This suggests that pulmonary measurements of RER give slightly greater rates of lipid oxidation and lower carbohydrate oxidation than is actually occurring within the working muscle. The lower whole body RER likely reflects a "watering down" effect from other non-exercising tissues that are predominately utilising lipid (and so a lower RQ).

Measurements of pulmonary $\dot{V}O_2$and $\dot{V}CO_2$ will also only reliably reflect the tissue O_2 uptake and CO_2 production during periods where the body's bicarbonate pool is stable. At rest and lower exercise intensities this assumption is likely valid with no substantial accumulation of hydrogen ions with lactate production closely matched by clearance (oxidation / gluconeogenesis). Under conditions of increased glycolytic flux, at exercise intensities above the maximal lactate steady state there are likely shifts in the acid base balance, with an excess of H^+ ions and lactate accumulating in the muscle. The increase in H^+ ions will be buffered by the bicarbonate pool, eventually producing non-oxidative CO_2, elevating the pulmonary $\dot{V}CO_2$, resulting in an artificial overestimation of any calculated rates of carbohydrate and underestimation of lipid oxidation. Romijn *et al* (Romijn et al. 1992) demonstrated the validity of indirect calorimetry to determine substrate oxidation by

comparing it to a breath ^{13}C to ^{12}C ratio technique which is independent of $\dot{V}CO_2$ (and thus not reliant on a stable bicarbonate pool). The authors reported no difference in the calculated substrate oxidation rates at 80-85%$\dot{V}O_2$max between the two techniques, suggesting indirect calorimetry is valid even at these relatively high exercise intensities.

Furthermore, it is typically assumed (as is the case in Chapters 4 & 5 of this thesis) that protein oxidation during exercise is minimal and substrate oxidation rates are calculated without a correction for protein oxidation. However, studies using isotopically labelled amino acids have revealed that branch chain amino acid (BCAA) oxidation actually increases during exercise, in-line with the overall increase in energy demand. Although the overall flux changes only marginally, with the increase in AA oxidation partially accounted for by a reciprocal attenuation in non-oxidative disposal of AAs (Millward et al. 1982). The increase in AA oxidation above resting values has been reported to be within the order of 50-400% (Millward et al. 1982; McKenzie et al. 2000), this however, only represents a 2-4% contribution to total substrate oxidation. Hypothetically, in well-nourished individuals in protein balance, with a typical 15% EI from protein (as was the case in Chapter 5), then the contribution of AA to resting EE can be assumed to be 15%. If this absolute contribution to EE remains constant, then even with a 15 fold increase in EE with exercise, AAs would only contribute 1% to total EE (Brooks 2012). Therefore, with the many complexities, uncertainties and costs involved in determining AA oxidation along with the likely minimal contribution to total EE, it was not measured in this thesis. Additionally, with endurance training further attenuating AA oxidation by ~25% (McKenzie et al. 2000) this offers further justification for not measuring AA oxidation in the trained runners in Chapter 5.

A further assumption of indirect calorimetry is that other metabolic processes that also consume O_2 and produce CO_2, such as gluconeogenesis, lipogenesis and ketogensis, do not substantially impact RER during exercise. Gluconeogenesis through the precursors lactate, pyruvate and glycerol, although consuming a net 4 molecules of ATP throughout the Cori cycle, do not involve gaseous exchange (Frayn 1983) and therefore do not impact RER or calculated substrate oxidation rates. However, gluconeogenesis from the major non-carbohydrate pre-cursor alanine (Felig et al. 1970), which requires the transfer of the amino group via glutamate into urea, consumes energy whilst fixating CO_2 into carbamoyl-phosphate, potentially influencing RER. The rate of gluconeogenesis during exercise is however believed to be relatively low in comparison to energy flux, providing around 25% of total glucose production during 90mins of moderate intensity exercise (Bergman et al. 2000; Trimmer et al. 2002). Gluconeogenesis does however become increasingly important as the duration of exercise becomes more prolonged (>4hrs) especially under fasted conditions, providing closer to 45% of total glucose production. Ahlborg *et al* (Ahlborg et al. 1974) estimated that up to 8% of glucose production under these conditions was provided by the gluconeogenic pre-cursor alanine, although still only contributing < 1% to total energy expenditure. Thus it is unlikely that gluconeogenesis during exercise has a substantial impact upon calculated rates of substrate oxidation.

The process of lipogenesis is only thought to occur during periods of positive energy balance with excessive carbohydrate consumption (Acheson et al. 1988) which can raise CO_2

production giving an RER >1. Exercise typically incurs a state of negative energy balance, and so rates of lipogenesis are thought to be minimal and thus not substantially impacting calculations of substrate oxidation during exercise.

In an O_2 consuming process, the condensation of acetyl-CoA units liberated during beta-oxidation of FAs produces ketone bodies such as acetoacetate. When this process of ketogenesis occurs in excess of ketone body oxidation, ketone bodies can either accumulate in extracellular fluid or can be excreted in the breath. During prolonged moderate intensity exercise when plasma NEFAs and rates of lipid oxidation are elevated, a surge in plasma ketone body concentration is also frequently observed, and it is these non-oxidised ketone bodies that can thus impact calculated rates of lipid or carbohydrate oxidation. In a similar manner to lactate, ketone bodies are almost entirely dissociated at physiological pH, possibly displacing a similar amount CO_2 from bicarbonate (Lusk 1919). If ketone body concentrations are measured along with estimations of the distribution volume for them to dissipate into (Keller, Sonnenberg, and Stauffacher 1981), then an estimation of ketogenesis and its effect on $\dot{V}O_2$ can be made with changes to substrate oxidation calculations made.

Thus, with appropriate considerations given to the assumptions and potential limitations of indirect calorimetry, it can be used as a valid measurement of substrate oxidation during exercise below 80% $\dot{V}O_2$max.

3.1.3 Measurement techniques

3.1.3.1 Oxycon Pro

Breath-by-breath measurements were made during exercise in both Chapter 4 and 5 by using an open circuit online automated gas analysis system (Oxycon Pro, CareFusion UK Ltd, Basingstoke, UK). Subjects either wore an airtight face mask (7450 V2, Hans Rudolph, Missouri, USA) or breathed through a mouthpiece whilst wearing a nose clip (to eliminate nasal breathing), with inspired and expired air passing through a turbine and volume transducer to determine $\dot{V}E$. Expired air was also sampled through a twin-tube to measure O_2 and CO_2 through paramagnetic and infrared analysers, respectively. Immediately prior to use the Oxycon Pro was calibrated, firstly for the volume measurement using a 3L calibration syringe, with this known volume passing through the lightweight bi-directional digital volume transducer. The volume transducer holds a small turbine and integrates an opto-electric reading unit. The blades of the turbine break an infrared beam detected by the reading unit which is counted by the computerized system thereby determining the rate of airflow. The integrated gas analysers were automatically calibrated using certified reference gases (5.07% CO_2, 14.79% O_2) (BOC Gases, Surrey, UK). Computer software synchronised the information collected on the expired O_2, CO_2 and the $\dot{V}E$ and averages data every 8 breath cycles, with this data exported for analysis in 10sec averages.

The validity of the Oxycon pro for measurement of $\dot{V}E$, $\dot{V}O_2$ and $\dot{V}CO_2$ was determined on a regular basis by direct comparison to measurements of expired gases collected in Douglas bags. The comparisons were made at various submaximal exercise intensities ranging

between 20-75% $\dot{V}O_2$max). Once an assumed steady state had been achieved (typically between 5-10mins), a 3min sample of expired air was taken using the Oxycon pro followed immediately by three separate 30-60sec samples in separate Douglas bags followed by another 3 minute sample using the Oxycon pro. The average of the three Douglas bags measurements were compared to the average of the Oxycon Pro. The volume of expired gas from the Douglas bags was measured using a dry gas meter (Harvard, Kent, UK) and the temperatures measured with a digital thermistor (model C, Edale Instruments, UK). The fraction of expired O_2 / CO_2 was assessed using paramagnetic and infrared analysers, respectively (Servomex 1400B4, Sussex, UK) which was calibrated prior to use, in a two-step process; firstly the sensors were zeroed with 100% Nitrogen gas (BOC Gases, Surrey, UK), before the gas-span was determined using high and low certified reference gases (High, O_2 17.9%, CO_2 2.0%, N_2 80.1%; Low, O_2 15.04%, CO_2 5.02%, N_2 79.94%) (BOC Gases, Surrey, UK). The expired gases in the Douglas bags were measured as close to collection time as possible and no longer than 5mins post sample to minimise any potential losses of gas especially CO_2 that may compromise the measurement (Hopker et al. 2012). The mean coefficient of variation for both the Douglas Bag methodology and that of the Oxycon Pro and the average percentage difference between the methods can be seen in Table 3-1. Additionally, the Oxycon pro was validated against a metabolic simulator (Jaeger, Germany). The simulators mass flow controllers, valve system and pump enable gases of known concentration to be injected through the simulator at 4 flow rates corresponding to 20, 40, 80 and 160l/min that should correspond to an RER of 1.0 at each flow rate. The results of an example simulation can be seen in the Appendix 7.1

Table 3-1. Reliability measurements

Measurement System	Average coefficient of variation (%)			
	$\dot{V}O_2$	$\dot{V}CO_2$	$\dot{V}E$	RER
Oxycon Pro	3.0	3.2	3.2	2.5
Douglas Bags	1.8	2.4	1.2	2.3
	Average percentage difference			
	$\dot{V}O_2$	$\dot{V}CO_2$	$\dot{V}E$	RER
Oxycon vs Douglas Bag	4	5	12	1

3.1.3.2 GEM

Resting breath measurements were made for the determination of RMR in chapter 5 using an open circuit GEM Indirect Calorimeter (GEMNutrition Ltd. Cheshire, UK). Subjects lay in a supine position with an opaque plastic hood positioned over the head with a sampling tube connected to the GEM gas analyser. Expired air is drawn through the tubing to a thermal mass flow meter to measure gas flow rate, where it is de-humidified and passed through a high performance paramagnetic oxygen analyser and infra-red CO_2 analyser to measure the concentration of the respective gases which are then integrated with the flow rate using the online software to determine $\dot{V}O_2$and $\dot{V}CO_2$. The integrated gas analysers were automatically calibrated prior to each use by using certified reference gases (1.00% CO_2, 21.00% O_2, balance Nitrogen) (BOC Gases, Surrey, UK).

The GEM gas analyser has a more sensitive CO_2 analyser (repeatability 0.02% abs) than that found in the Oxycon Pro (0.05% abs). Considering the much lower flow rates of $\dot{V}O_2$ and

$\dot{V}CO_2$ at rest compared to exercise, the greater sensitivity of the GEM gas analyser makes this a more appropriate system capable of detecting subtle differences in expired gases and thus calculated substrate oxidation rates during resting conditions.

3.2 Resting Metabolic Rate

Resting metabolic rate (RMR) represents the obligatory energy requirements whilst at complete rest (expressed as kcal/day or MJ/day), it is the energy required to maintain essential metabolic process such as the contraction of cardiac or respiratory muscle or the energy required for maintain active solute pumps (Fiehn and Peter 1971). In most individuals, especially those with low levels of physical activity, RMR represents the largest component of daily energy expenditure (60-75%) (Harris and Benedict 1918). Whilst RMR has been shown to vary between individuals, there are several known key determinants, making it possible to predict RMR within reasonable accuracy of a measured value using easily obtainable anthropometric and descriptive variables. In Chapter 4, RMR was estimated using the predictive equations of Harris-Benedict (see below) (Harris and Benedict 1918) whereas in Chapter 5 it was either measured via indirect calorimetry (*n* =8) or estimated using the Harris-Benedict equation (*n* =7). Ideally a measured RMR value would have been made throughout both experimental chapters with this accounting for individual variation and enabling greater precision in the calculations of energy expenditure. However, for logistical reasons, and constraints in the testing time with the relatively large sample size in Chapter 4, an estimate of RMR from a prediction equation was considered an appropriate compromise with any small errors in the estimation at least being consistent between

individuals. The Harris-Benedict equation is well established, shown to be more valid than other equations (Flack et al. 2016) and the equation is also based upon RMR values measured from a cohort fairly representative of the majority of individuals from Chapter 4 (young, healthy Caucasian).

In Chapter 5, the much smaller study sample size made it possible to measure RMR through indirect calorimetry using the Weir equation without correction for urinary nitrogen (Weir 1949):

Non protein corrected Weir Equation for RMR:

RMR (kcal/day) = ([3.941 * $\dot{V}O_2$(L/min)] + [1.106 * $\dot{V}CO_2$ (L/min)]) * 1440(min/day)

Unfortunately, due to equipment failure, RMR measurements were only available on 8/15 participants in Chapter 5, with the Harris-Benedict equation chosen to estimate RMR in the remaining 7 participants for consistency with chapter 4. An independent samples t-test was used to compare the measured RMR values (1586 $\pm$ 221 kcal) to the Harris-Benedict calculated values (1514 $\pm$ 219 kcal) and did not significantly differ (p=0.28) showing a good level of agreement.

Harris Benedict Equation for estimating RMR:

Men: 66.5 + (13.75*weight (kg)) + (5.003*height (cm)) – (6.755*age (yrs.))

Women: 655.1 + (9.563*weight (kg)) + (1.850*height (cm)) – (4.676*age (yrs.))

Besides achieving a steady state in expired gases, there has generally been a lack of consistency in the protocol or approach taken to determine RMR, despite recommendations available (Compher et al. 2006). Protocols previously used have varied in complexity from a simple average over a defined period of time after steady state, to the average with the lowest coefficient of variation (Shook et al. 2015), or a period with <10% coefficient of variation (CV) for $\dot{V}O_2$/ $\dot{V}CO_2$, or the lowest value taken from four 5 min periods accepted as stable (within 100 kcal/ day) (Walhin et al. 2013). When RMR was measured in Chapter 5, it was measured over a 30min period, the first 10 mins and last 2 mins of which were discarded, with the average of the remaining 18mins used to determine the RMR provided the CV for $\dot{V}O_2$/ $\dot{V}CO_2$ was <10%.

3.3 Dietary Analysis

Obtaining accurate, reliable and valid measures of habitual dietary intake is a notoriously difficult process (for a comprehensive review see (Bingham 1987)). No currently employed methodology is considered to be a gold-standard approach, although a weighed food diary approach is thought to provide the most accurate and detailed nutritional information provided subject compliance to the burdensome methodology is high. In this thesis two methods were employed, in Chapter 4 detailed habitual dietary intake data was required, therefore a 4-day weighed food diary approach was used (see Appendix 7.2 for example record), whereas a 24 hr recall method was used in Chapter 5 to minimise subject burden whilst still providing reliable, detailed, dietary data. However, with the well-recognised inaccuracies and systematic underestimation of actual intake with all self-report methods

(IOM 2000 Dietary Reference Intakes: Applications in Dietary Assessment. Washington, DC: National Academy Press.), these limitations should be considered when interpreting the data.

3.3.1 Weighed Food Diary

Although it could be argued that a 4-day food diary might not be considered to be reflective of habitual dietary intake, this duration of measurement period was taken as a pragmatic approach to enable a sufficient level of detail on dietary intake without being overly cumbersome on a large sample of participants. Additionally, reliable dietary data in the period closest to when the metabolic testing commenced was of primary importance, and with subject compliance to dietary recording reduced after more than 4 days (Gersovitz, Madden, and Smiciklas-Wright 1978), this duration of recording was chosen.

Each participant was provided with detailed instructions both verbally and in written form to carry out a weighed food diary (see Appendix 7.2). Participants were supplied with two sets of digital scales, one pocket sized to encourage and allow accurate weighing when away from home and a second more traditionally sized kitchen scales. Subjects were trained how to operate the scales and advised where possible on procedures to try and limit instances that might not reflect their normal dietary practice, with emphasis on the weighing everything and providing a high level of detail. When weights of foods / drinks were not possible the subjects were required to estimate and record in household items the quantity of food / drink consumed. All relevant details regarding dietary intake were obtained in the

diary including, specific food names/brands, individual food weights, cooking methods, leftovers etc. All food diaries were checked for completeness with the participant, using open ended probing questions to verify that no items were missed and all detail needed for dietary assessment was captured. All recorded food items, meals and homemade recipes were later inputted and analysed using the computer software Dietplan 6.70.67 (Forestfield Software Ltd. Horsham, West Sussex, UK), which makes assumptions for nutrient losses during the different cooking processes / technique used where applicable. In instances where a recorded food item was not included in the databases within Dietplan software, the data was obtained from the manufacturer and entered into the database to improve accuracy.

3.3.2 24 Hour Recall

In Chapter 5 the habitual dietary intake was assessed using the multiple pass 24hr recall technique on 3 separate occasions. Participants were assured that no judgement would be made on dietary data given, and were asked to be honest. An initial quick list of all foods and drinks consumed over the proceeding 24hr period was then made without interruption from the interviewer. This was then followed up with a list of commonly omitted foods and beverages, such as milk / sugar in coffee. Information was gathered on the time, location and occasion of the listed foods in an attempt to improve recall. A more detailed pass was then conducted whereby specifics on the consumed foods listed is obtained, including brands, cooking techniques and estimated portion sizes with use of the Foods Standards Agency Food Portion Sizes booklet (Nelson et al. 1997), with one final review through for any

forgotten foods. The data obtained were then analysed using the same computer software Dietplan 6.70.67 (Forestfield Software Ltd. Horsham, West Sussex, UK).

3.4 Physical Activity / Energy Expenditure

The assessment of habitual physical activity levels for the determination of energy expenditure is tremendously difficult because of the wide variability in lifestyles (Thompson and Batterham 2013). Although the gold standard method of doubly labelled water method largely negates this problem and so would have been useful for capturing energy expenditure in Chapters 4 and 5 it was unfortunately not available and so the most accurate methods available considering the study sample sizes, subject burden and primary objective of the measurement were employed.

3.4.1 Activity Diary

In Chapter 4, self-reported physical activity level was calculated using a factorial approach (Manore, Meyer, and Thompson 2009) from self-reported data recorded using a physical activity diary adapted from Bouchard *et al* (Bouchard et al. 1983) (see appendix 7.3). Over 4 days, subjects were required to record their level of physical activity every 15 minutes using a code provided from a 12-point scale, each with a designated activity factor, corresponding to; rest (1.0), very light (1.5), light (2.5), moderate (5.0) and heavy (7.0) ((US) 1989). An average daily activity factor was calculated, weighted by the amount of time spent at each of the assigned codes per day, Total daily energy expenditure was estimated by multiplying the weighted activity factor value by RMR estimated using the Harris-Benedict equation (Harris

and Benedict 1918). A similar methodology to estimate EE has been validated in 15yr old adolescents against double labelled water (mean difference in calculated energy expenditure 1.2%) (Bratteby et al. 1997).

The four days of activity recording used in Chapter 4, is unlikely to be truly reflective of habitual activity levels, being too short in duration to sufficiently capture the variability within an individual's habitual lifestyle. However, a primary reason for the measurement of energy expenditure in Chapter 4 was to determine the contribution of energy balance in the variability in MFO. In this regard, the methodology employed was likely of sufficient detail and duration, with the acute energy balance likely most relevant, even if the act of monitoring activity levels altered behaviour (Hawthorne effect) as is often reported and influenced the estimate made (Wickström and Bendix 2000). Typically, self-reported physical activity levels are overestimated when they have been compared to objectively made measurements (IOM 2005; Loney et al. 2011), with high subject compliance crucial to the validity of data. A positive association is also often reported between BMI and the degree of over-reporting of subjectively measured physical activity (Warner et al. 2012) the exclusion of individuals with a BMI >30 potentially attenuated the impact of this limitation. In light of the subjectivity and limitations in methodology employed the data collected must be interpreted with a degree of caution.

3.4.2 Combined Heart Rate and Accelerometer

In Chapter 5 physical activity energy expenditure was determined objectively through the use of an Actiheart monitor (CamNtech, Cambridge, UK). This small monitoring device was

attached to the chest by long-term ECG pads (Red Dot solid gel electrodes, 3M, Bracknell, UK) and simultaneously measures heart rate and uni-axial accelerometer movements. Using a branched equation model (Brage et al. 2004) these combined measurements are used to determine energy expenditure, negating the pitfalls of either method used in isolation (Crouter, Churilla, and Bassett 2007). The Actiheart unit and the branched equation model used have been described in more detail previously (Brage et al. 2005; Brage et al. 2004). Individual heart rate-$\dot{V}O_2$ calibration curves established the individual relationship between heart rate and energy expenditure enabling the use of the "Group Act/Ind HR+stress" energy model from the Actiheart software (Actiheart software version 4.0.116, CamNtech, Cambridge, UK), with sleeping and max heart rate, $\dot{V}O_2$max and RMR also entered to account for individual variability, improving estimates of energy expenditure (Assah et al. 2011). Recordings were made in 30 sec epochs throughout the wearing time, and the raw data was cleaned and processed using the Actiheart 4 software. Total daily energy expenditure was the sum of RMR, Actiheart derived physical activity energy expenditure and an estimated diet induced thermogenesis (10% daily EI) (Westerterp 2004).

The Actiheart monitor is able to reliably capture daily energy expenditure, showing good agreement with the criterion method in free living individuals (doubly labelled water) in both adults (Assah et al. 2011) and children (Zamora-Salas and Laclé-Murray 2015). The energy expenditure calculated by the Actiheart during more discrete everyday activities (MET<4.3) such as walking or sweeping also compare well (6% under estimation) to those measured via indirect calorimetry, with no fixed or proportional bias (Thompson et al. 2006). Importantly considering the study population (predominantly runners) in Chapter 5, the Actiheart

derived energy expenditure values also compare well to indirect calorimetry under more strenuous activities such as running (Barreira et al. 2009; Brage et al. 2005).

3.5 Blood collection and analysis

3.5.1 Blood Collection

In Chapter 5 venous blood samples were taken both at rest and during exercise to offer insights to the metabolic effect of the study diets. Venous blood samples were drawn through an indwelling cannula (20g IV catheter, BD Venflon, Plymouth, UK) inserted into an antecubital vein connected to a 150 cm polyethylene extension line (V-Green I.V. Extension Line, Vygon, Swindon, UK). A 3-way stopcock (BD Connecta, Plymouth, UK) was attached to the extension line, allowing blood samples to be drawn whilst the participant was running on the treadmill without the need for stopping or substantial changes in running gait. Samples were drawn using a 10ml syringe and decanted into vacutainers containing either, EDTA, lithium heparin, or no additive to allow clotting, depending on the analysis required. EDTA and lithium heparin tubes were immediately stored on ice, with serum tubes left to clot at room temperature, after which all tubes were centrifuged at 1361g (3000rpm) for 15mins at 4°C. Aliquots containing plasma or serum were then flash frozen in liquid nitrogen and stored at -80°C until analysed.

Although valuable metabolic insights can be gained from the venous blood samples taken, it is pertinent to consider that these samples only provide a snap-shot of the metabolic

processes at the time of sampling. Without a measure of the Ra or Rd of a metabolite it is impossible to draw conclusions on the flux of relevant metabolites which may increase substantially during exercise without changes observed in total blood concentration. Additionally, the concentration of hormones and metabolites in venous blood samples can potentially differ to arterial blood, having specific metabolites extracted and others added and so might not reflect what was available to the muscle, but what was expelled or not extracted. However measuring arterial blood comes with an inherent risk and at high flow rates observed during exercise the differences between the two is minimised (Wallis et al. 2007), thus venous blood samples were obtained in this thesis as is common practice in the field.

3.5.2 Targeted metabolite analysis

The concentration of plasma glucose and lactate served as markers of carbohydrate metabolism, with an increase in lactate characteristic of a greater rate of carbohydrate flux during exercise. The circulating concentration of NEFA and glycerol were representative of markers of lipid metabolism. Under the provision that all lipolysis of TAG is complete and released into the circulation without re-esterification, then plasma glycerol concentration serves as a marker of whole body lipolysis. Although these assumptions may not entirely hold true (Hall et al. 2002; Van Hall et al. 2002; Landau 1999) glycerol Ra is highly responsive to lipolytic activators or suppressors and so is useful at least as a marker of the minimal rate of whole body lipolysis (Coggan 1999). Serum TAG concentration and the lipoprotein profiles

were also measured to investigate the impact of the study diets from a health perspective, with these established markers for the development of cardiovascular disease.

The concentration of targeted metabolites (glucose, lactate, NEFA, TAG, glycerol, total cholesterol, LDL-C and HDL-C) were determined in duplicate using enzymatic catalysed colorimetric assays, measured on a semi-automatic spectrophotometric analyser (ILAB 650 Clinical Chemistry Analyser, Instrumentation Laboratory, Cheshire, UK). Following incubation, the absorbance value of each assay is measured and the analyte concentration determined by the linear relationship between concentration and absorbance of specific frequency light waves. Both the intra and inter-assay co-efficient of variation for the different analytes can be seen in Table 3-2.

Table 3-2 Analyte and description of assay including variation in measurement technique

Analyte	Assay (commercially available kit name and supplier)	General principle (enzymatically)	Coefficient of variation (%)	
			Intra-assay	Inter-assay
Plasma glucose	Glucose Oxidase, (Instrumentation Laboratories, Cheshire, UK)	Trinder methodology: glucose oxidase / peroxidase	1.5	5.9
Plasma Lactate	L-Lac, (Randox, London, UK)	Lactate Oxidase / Peroxidase	2.0	3.9
Plasma NEFA	NEFA, (Randox, London, UK).	Acyl CoA Synthetase / Acyl CoA Oxidase / Peroxidase	3.3	3.1
Plasma glycerol	GLY, (Randox, London UK)	Glycerol kinase / peroxidase and glycerol phosphate oxidase	2.8	5.9
Plasma triglyceride	Triglycerides, (Instrumentation Laboratories, Cheshire, UK)	Lipoprotein Lipase / Glycerol Kinase / Glycerol Phosphosphate Oxidase / Peroxidase	1.3	N/A
Serum total cholesterol	Total cholesterol, (Instrumentation Laboratories, Cheshire, UK)	Bichromatic analysis, modification of the method of (Allain et al. 1974) cholesterol Esterase / cholesterol Oxidase / Peroxidase)	4.4	N/A
Serum HDL-C	HDL, (Instrumentation Laboratories, Cheshire, UK)		3.7	N/A
Serum LDL-C	LDL, (cholesterol Instrumentation Laboratories, Cheshire, UK)	cholesterol Esterase / cholesterol Oxidase	3.8	N/A

Coefficients of variation are based on analysis of 20 duplicate plasma samples ran across 2 assays, if N/A analysis was completed within a single assay.

3.5.3 Hormone analysis

Serum insulin concentration was determined in duplicate by radioimmunoassay using a commercially available kit (HI-14 K Human Insulin, Millipore, Hertfordshire, UK). A fixed concentration of ^{125}I-labelled human insulin antigen was incubated with a constant dilution of human insulin antiserum such that the number of antigen binding sites on the antibody is limited. Upon mixing with the sample, the unlabelled insulin antigen then competes for the limited and constant number of binding sites on the antibody, with the amount of ^{125}I-labelled antigen bound to the antibody being inversely proportional to the concentration of insulin in the sample. After separating the antibody-bound from the free labelled tracer a gamma counter measures the level of radioactivity. A standard curve was set up with 6 serial dilutions of standard unlabelled 200 µU/mL antigen and from this curve the amount of antigen in unknown samples can be calculated. This analysis was undertaken by Dr Edward Chambers at Imperial College London due to a substantial saving in cost, safety (regarding the radioactivity) and greater sensitivity compared to the alternative of an in-house analysis through commercially available ELISA kits. The average intra-assay coefficient of variation was 2.8% based on 96 duplicate samples with the inter-assay coefficient of variation 0.5% based on 12 samples ran across 2 assays.

Serum oestrogen and progesterone concentrations were measured to confirm in which phase of the menstrual cycle the subjects in chapter 5 were tested. In separate analyses the hormone concentrations were quantified by competitive principle immunoassays using a Roche e602 unit on a Cobas 8000 modular analyser (Roche Diagnostics Ltd, Rotkreuz,

Switzerland). Both hormones were incubated with specific antibodies and a labelled hormone derivative that competes equally for the antibody biding site. The amount of the labelled hormone derivative bound in the solid phase is inversely proportional to the quantity of oestrogen or progesterone. The bound particles are magnetically captured onto an electrode which has a voltage applied inducing chemiluminescent emission which is then measured by a photomultiplier. This analysis was undertaken by GF and Nazir Ahmed at the Queen Elizabeth Hospital Birmingham because of substantial cost savings compare to the alternative of an ELISA, plus greater detection limits for oestrogen, which were necessary given the anticipated low concentrations in the phase of the cycle testing was planned to commence in (follicular phase). The inter-assay coefficient of variation for oestrogen (0.73%) and progesterone (2.0%) were based on analysis of 8 (oestrogen) and 6 (progesterone) serum samples ran across 2 assays.

3.5.4 Metabolomics

In Chapter 5, system level mechanistic insights into the metabolic effects of the study diet were obtained using non-targeted metabolomics. Metabolomics uses mass-spectrometry to identify and quantify small (<1500 Da) metabolites in the metabolome (Hollywood, Brison, and Goodacre 2006). By studying the metabolome (the complete set of small molecule chemicals) the overall impact of the study diets is measured, with the metabolome being downstream of other biochemical species and biochemical information flowing from genome to transcriptome to proteome to metabolome. This top-down approach to studying metabolism, enables the detection and quantification of more than 4000 metabolites

thereby generating huge amounts of exploratory data at the whole body level, offering a sensitive measure of the biological phenotype. This untargeted approach of metabolomics can better distinguish individual phenotypes than more conventional measures of only a small set of metabolites (Assfalg et al. 2008).

There are several different analytical platforms available for the detection, identification and quantification of a range of different metabolites, such as UHPLC –MS or nuclear magnetic resonance spectroscopy available. Each platform comes with its own strengths and weaknesses. Due to the vast chemical diversity of metabolites, varying in physico-chemical properties and in concentration, no one methodology can capture the entire metabolome. There is also a lack of well-established and standardised methods of procedures for the different mass-spec approaches in identifying and discriminating metabolites, which can make comparisons difficult.

An untargeted UHPLC-MS approach was considered to be the most appropriate methodology for the work in this thesis. This approach captures a holistic and unbiased picture of metabolism relevant for the exploratory nature of this study, with its ability to detect lipid soluble and insoluble compounds across wide spectra of mass. A more detailed description of the UHPLC-MS approach used within this thesis is provided in the appendix (7.6).

4 DETERMINANTS OF THE INTER-INDIVIDUAL VARIABILITY IN THE CAPACITY FOR FAT OXIDATION DURING EXERCISE: IMPACT OF DIET, BIOLOGICAL SEX, MENSTRUAL CYCLE AND HORMONAL CONTRACEPTIVES

4.1 Dietary intake independently predicts the maximal capacity for fat oxidation during exercise and does so in a sex-specific manner

4.1.1 Introduction

The capacity to oxidize fat (fat oxidation, FAT-OX) as a fuel is important for metabolic health, weight management and body composition. For instance, skeletal muscle of patients with type 2 diabetes is associated with an impaired ability to oxidize fat (Kelley and Simoneau 1994). Further, a high respiratory quotient (RQ), indicative of low FAT-OX relative to carbohydrate oxidation (CHO-OX), is predictive of both future body mass gain (Zurlo et al. 1990; Marra et al. 2004; Shook et al. 2015) and regain of fat mass (FM) after diet-induced reductions in body mass (Ellis et al. 2010). Exercise acutely increases both energy expenditure and FAT-OX and the capacity to oxidize fat during exercise is related to daily FAT-OX and insulin sensitivity (Robinson et al. 2015). Further understanding factors that are associated with FAT-OX during exercise could, therefore, help to optimize the use of physical activity for maintenance of metabolic health, body mass and composition (Brooks et al. 2004).

The pattern of fuel utilization during exercise performed under a variety of experimental conditions has been well characterized. (Brooks and Mercier 1994; Venables, Achten, and Jeukendrup 2005; Achten, Gleeson, and Jeukendrup 2002; Romijn et al. 1993; Volek et al. 2016). Despite this, substantial inter-individual variability in energy substrate partitioning and the maximal rate of fat oxidation (MFO) during exercise has been described (Achten and Jeukendrup 2003; Venables, Achten, and Jeukendrup 2005; Volek et al. 2016). A previous study attributed 35% of the inter-individual variability in MFO to aerobic fitness ($\dot{V}O_2max$), Self-Reported Physical Activity Level [SRPAL], body composition (Fat Free Mass [FFM], FM) and sex (Venables, Achten, and Jeukendrup 2005). Dietary intake could contribute to the observed inter-individual variability in MFO, although its relative influence has not been quantified.

A high fat diet can increase FAT-OX during exercise whereas an isoenergetic high CHO diet can reduce it (Helge, Wulff, and Kiens 1998; Volek et al. 2016; Phinney et al. 1983). However, the outcomes of studies exploring widely divergent macronutrient intakes may not be applicable to the habitual dietary patterns reported by the majority of the population (Statistics 2016; Bates et al. 2014). Previously, a significant inverse relationship between self-selected dietary fat intake and exercising respiratory exchange ratio (RER) has been reported (Goedecke et al. 2000). This observation provides an important insight but the relatively small sample size, use of an exercise-trained cohort and limited range of exercise intensities studied precluded full exploration of the association of diet on FAT-OX across a broad spectrum of active individuals.

Understanding the independent contribution of diet relative to other known contributors to FAT-OX represents an important step. Diet is a modifiable variable, which could enhance FAT-OX during exercise. As MFO occurs at moderate intensities, diet could influence FAT-OX at exercise intensities consistent with current public health recommendations, with broad implications for public health – regarding both body composition and metabolic health. Therefore, the primary aim of this study was to determine the extent to which diet independently predicts the inter-individual variability in MFO during exercise in healthy young men and women. Additionally, it has been reported that women exhibit greater rates of FAT-OX than men during exercise (Tarnopolsky et al. 1990), yet the role of biological sex, independent of other factors relevant to substrate oxidation [e.g., $\dot{V}O_2$max, SRPAL, body size-related variables (Venables, Achten, and Jeukendrup 2005)] on MFO, has not previously been assessed, so will be herein.

4.1.2 Methods

4.1.2.1 Participants

Between January 2013 and March 2014, three hundred and seventy seven individuals were assessed for their eligibility to participate in the study, with 364 (181 men and 183 women) meeting the inclusion criteria. Data collection was completed by March 2014. Participants were recruited from the surrounding (West Midlands, UK) local community by postal notices, emails and word of mouth. Participants were excluded from taking part if they were: <18 or > 45 years old, BMI <18.8 or > 29.9 kg/m^2, > 192cm in height (maximum Dual-Energy X-ray Absorptiometry [DXA] scanning height), taking any medication or supplements with

the potential to interfere with normal metabolism (e.g., beta-blockers, insulin, bronchodilators, anti-inflammatory agents, thyroxine), were completely sedentary, a current or recent (within 30 days) smoker, engaged in prolonged periods of food abstinence, pregnant, breast feeding or amenorrhoeic combined with not using hormonal contraception. Participants provided written informed consent in accordance with the Helsinki Declaration of 1975 as revised in 1983 to take part in the study that was approved by the National Research Ethics Service Committee East Midlands, Northampton, UK (Ref: 12EM0470). A total of 305 (150 men and 155 women) participants completed the study, which met the a priori objective to achieve a similar number to prior work (Venables, Achten, and Jeukendrup 2005). A flow chart of participant recruitment and involvement in the study is shown in Figure 4-1. Participants (*n* = 24) withdrew after providing consent for the following reasons; musculoskeletal injury preventing completion of exercise testing (*n* = 5), development of cold/flu like symptoms (*n* = 2), personal reasons unrelated to study (*n*= 4), uncomfortable with the exercise testing (*n*= 5), lost to follow up – i.e. not contactable (*n* = 8). Data from 34 subjects was excluded from the analysis as they were unable to fully comply with the study protocol. Data from one subject was excluded on statistical grounds (see Statistical Analysis).

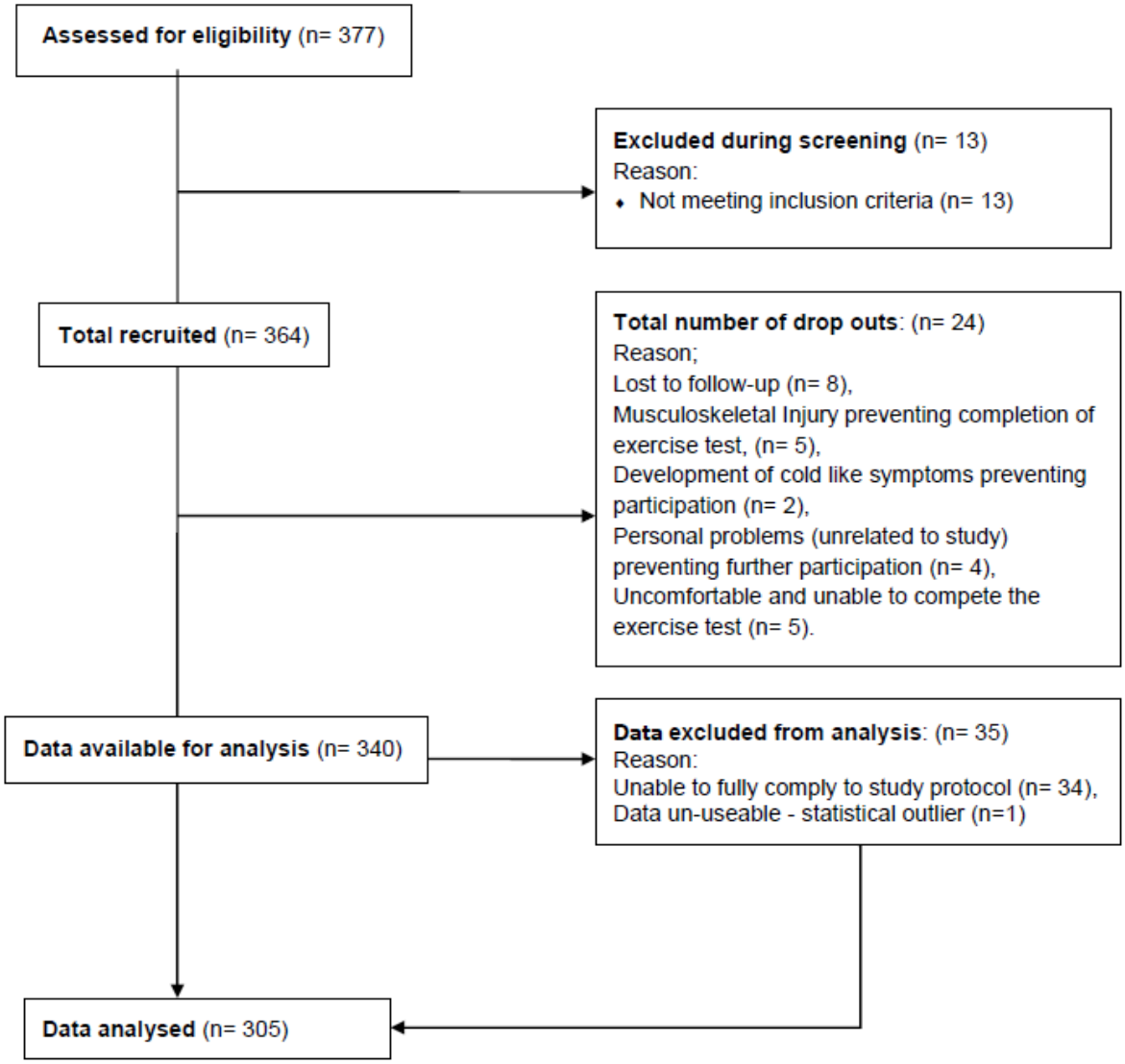

Figure 4-1 Flow chart of participant recruitment and involvement in the study

The characteristics of the 305 participants that completed the study are shown in Table 4-1. All volunteers were deemed to be healthy as assessed by a general health questionnaire, and self-assigned ethnicity via questionnaire. The women also completed a self-report questionnaire to estimate the menstrual cycle phase during which testing occurred and to

document hormonal contraceptive (HC) use (Follicular *n* = 57, HC *n* = 19; Luteal *n* = 82, HC *n* = 30; amenorrhoeic and taking HC *n* = 16).

4.1.2.2 Study Design

The current study followed a single-centre, cross sectional design with each participant attending the laboratory on two occasions separated by 5-10 days. At the first visit, demographic data were recorded before undertaking a familiarization exercise test (identical to that described below). Participants were provided with digital weighing scales and a food and physical activity diary that they completed during the four consecutive days before the second visit to the laboratory. They were instructed to maintain their normal dietary habits and physical activity levels during this time, with the exception of the day before the second visit where they refrained from strenuous physical activity and alcohol consumption. Following a 10-12hr overnight fast except for water, participants attended the laboratory for the second visit between 07:00-09:00 where anthropometric measurements and body composition were determined. Thereafter, participants completed an exercise test to determine submaximal exercise substrate utilization, MFO and $\dot{V}O_2$max using indirect calorimetry.

Table 4-1 Participant demographic characteristics, ethnicity, aerobic capacity and self-reported physical activity levels

Variable	All Subjects (*n* = 305)	Men (*n* = 150)	Women (*n* = 155)
Age (years)	25 ± 6	24 ± 7 (18-45)	25 ± 6 (18-45)
Height (m)	1.72 ± 0.09	1.78 ± 0.06*	1.66 ± 0.06
Mass (kg)	68.7 ± 11.1	76.0 ± 9.1***	61.6 ± 7.9
BMI (kg/m^2)	23.0 ± 2.0	23.9 ± 2.3***	22.2 ± 2.2
WC (cm)	78 ± 8	82 ± 7***	73 ± 7
Body fat (%)	24.7 ± 7.1	19.6 ± 4.8***	29.7 ± 5.1
Fat free mass (kg)	51.2 ± 10.8	60.1 ± 7.2***	42.6 ± 5.1
Fat mass (kg)	16.5 ± 5.0	14.8 ± 4.6***	18.2 ± 4.8
Visceral adipose tissue (g)	227 ± 123	274 ±118***	181 ±110
A/LB FM[2]	0.17 ± 0.06	0.20 ± 0.06***	0.14 ± 0.04
$\dot{V}O_2$max (L/min)	3.44 ± 0.83	4.11 ± 0.57***	2.80 ± 0.46
$\dot{V}O_2$max (ml/kg/min)	49.9 ± 8.0	54.4 ± 6.9***	45.6 ± 6.6
$\dot{V}O_2$max (ml/kg FFM/min)	67.1 ± 7.0	68.5 ± 7.0***	65.7 ± 6.7
SRPAL	1.57 ± 0.13	1.58 ± 0.14	1.56 ± 0.12
Ethnicity			
White (*n*)	230	109	121
Black (*n*)	12	4	8
Asian/Indian/Pakistani (*n*)	27	18	9
Chinese/Other Asian (*n*)	23	8	15
Mixed (*n*)	13	11	2

[1] All values besides self-reported ethnicity data are Mean ± SD,

FFM, Fat Free Mass, SRPAL, Self-Reported Physical Activity Level, $\dot{V}O_2$max, maximal oxygen uptake; WC, waist circumference; A/LB FM, abdominal to lower body fat mass ratio.

Significantly different from women (unless stated: independent *t* test, [2] Mann-Whitney U test) * = P<0.05; *** = P<0.001

4.1.2.3 Four-Day Dietary and Physical Activity Assessment

Participants were provided with comprehensive written and verbal instructions explaining how to complete a four-day weighed food diary. Digital weighing scales, one portable pocket sized (Swees, Digital Pocket Mini Gold, Kent, UK) and one standard sized (Macallen TM Digital LCD Electronic Kitchen Weighing Scales 10kg, UK), were provided to allow all food and drink consumed to be weighed to the nearest gram. Each food diary was checked by the first author (GF), with any uncertainties clarified by the participant. The diaries were analyzed by GF using Dietplan 6.70.67 (Forestfield Software Ltd. Horsham, West Sussex, UK) to produce a comprehensive report of energy and nutrient intake. When a consumed food item was missing from the database, the nutritional data were located from the manufacturer and entered manually.

An adapted version of the physical activity record designed by Bouchard and colleagues (Bouchard et al. 1983) was used to estimate energy expenditure using the factorial approach (Manore, Meyer, and Thompson 2009). Briefly, subjects were required to record their level of physical activity every 15 minutes using a code provided from a 12-point scale, each with a designated physical activity level (SRPAL) value. A daily SRPAL value was determined from the total amount of time spent at each of the assigned codes per day. Total daily energy expenditure was estimated by multiplying the obtained daily SRPAL (1989) value by RMR estimated using the Harris-Benedict equation (Harris and Benedict 1918), with energy balance then calculated as energy expenditure minus energy intake. All energy expenditure,

energy balance and dietary intake data were averaged for the four-day measurement period.

4.1.2.4 Anthropometry and Body Composition Assessment

After voiding and whilst wearing minimal clothing, participants were weighed to the nearest 10g (OHaus, Champ II scales, USA) and height was measured to the nearest cm (Stadiometer, Seca, UK). Waist circumference was measured to the nearest mm with the tape measured midway between the uppermost border of the iliac crest and the lower border of the costal margin (rib cage). Body composition was determined by DXA (Hologic, Discovery QDR W series, Crawley, West Sussex, UK), with a manufacturer-recommended phantom scan performed daily for calibration and quality control assurance. As well, the abdominal to lower body FM (A/LB FM) ratio was determined from the DXA scan in a similar manner to that previously described (Isacco et al. 2013) differing only in the use of automated versus manual determination of abdominal fat (APEX version 4.0, Hologic Inc., Bedford, Massachusetts, USA).

4.1.2.5 Exercise Test

Participants were familiarized with the exercise test during Visit 1 to ensure that during Visit 2 the physiological and metabolic responses measured were as near normal and maximal as possible and not overly influenced by the performance of a novel task. The motorized treadmill (PPS 70sport-I, Woodway, Weil am Rhein, Germany) / HP cosmos, Quasar, Nussdorf-Traunstein, Germany) based exercise test was adapted from that used previously

by Achten and colleagues (Achten, Venables, and Jeukendrup 2003). The test commenced at a speed of 3.5 km/h and a gradient of 1% (to reflect the oxygen cost of outdoor running (Jones and Doust 1996)), and the speed was increased by 1 km/h every 3 min until a RER of 1.00 was reached and FAT-OX was therefore negligible thereafter (Jeukendrup and Wallis 2005). The treadmill speed was then kept constant with the gradient increasing by 1% every minute until volitional exhaustion to determine $\dot{V}O_2$max within the same protocol. Heart rate was recorded continuously by telemetry using a heart rate monitor (Polar S610i, Polar Electro Ltd, Oy, Finland). Environmental conditions during testing were: relative humidity 45±7%; temperature 20±2°C. An electronic fan was positioned behind participants for use upon request.

A face-mask (7450 V2, Hans Rudolph, Missouri, USA) was securely fitted, and breath-by-breath respiratory measurements (minute ventilation, $\dot{V}E$; oxygen consumption, $\dot{V}O_2$; carbon dioxide production, $\dot{V}CO_2$) were recorded throughout the test using an automated gas analysis system (Oxycon Pro, CareFusion UK Ltd, Basingstoke, UK). The gas analysers were calibrated immediately before each exercise test according to the manufacturers recommendations using calibration gases (5.07% CO_2, 14.79% O_2) (BOC Gases, Surrey, UK), and the volume transducer was manually calibrated with a 3 litre bi-directional syringe (Jaegar, Wuerzberg, Germany). The highest rolling 60 second average $\dot{V}O_2$measurement was considered to be maximal ($\dot{V}O_2$max) if two of the three following conditions were met: 1) a plateau (an increase of ≤2 mL/kg/min) in $\dot{V}O_2$with further increasing workload; 2) a heart rate within 10 beats/min of the age-predicted maximum (for males, 220 beats per minute –

age, for women 206 beats per minute – 0.88(age) (Gulati et al. 2010)); and 3) a respiratory exchange ratio of >1.1. If a plateau in $\dot{V}O_2$did not occur then a $\dot{V}O_2$peak value was obtained, defined as the highest $\dot{V}O_2$value averaged over a 30-second period. The average $\dot{V}O_2$and $\dot{V}CO_2$ during the final minute of each 3-minute submaximal stage of the exercise test was used to calculate FAT-OX and CHO-OX using the stoichiometric equations of Frayn (Frayn 1983) under the assumption of negligible urinary nitrogen losses. The highest attained rate of FAT-OX was identified as the MFO, and the exercise intensity (i.e., % $\dot{V}O_2$max) associated with this rate was identified as the FatMax (Achten, Gleeson, and Jeukendrup 2002). Using Matlab (MathWorks Matlab 2011a, Natick, Massachusetts, USA) CHO and FAT-OX rates were determined for each 5% increment of 35-85% $\dot{V}O_2$max by interpolation (1000 points) between subsequent recorded data points and logging the oxidation value nearest to the increment of $\dot{V}O_2$max.

4.1.2.6 Statistical Analysis

Data were analysed using the SPSS statistical package for Windows, version 20.0 (SPSS, Chicago, IL, USA) and the R statistical software package version 3.3.0 (R Foundation for Statistical Computing, Vienna, Austria). Data were checked for normality using distribution plots and the Kolmogorov-Smirnov test. Differences in CHO and FAT-OX rates between men and women across different exercise intensities were tested with a mixed ANOVA. Differences between men and women in baseline characteristics that were normally distributed were assessed using independent sample t-tests, with Mann-Whitney U tests

used for data non-normally distributed. Significant sex-based differences were found across most baseline characteristics (Table 4-1 &).

Table 4-2). Therefore, all independent variables were mean-centred by sex. This transformation allowed tests of the effects of sex independent of sex-related differences in baseline characteristics relevant to substrate oxidation (e.g., aerobic fitness, body-size related variables and diet). Analyses employed hierarchical multiple regression, with previously identified variables (FM, FFM, sex, SRPAL, $\dot{V}O_2max$) (Venables, Achten, and Jeukendrup 2005) entered on step 1 and macronutrient variables entered on step 2 to quantify the independent association of each variable with MFO expressed in absolute terms (g/min) and relative to fat free mass (mg/kg FFM/min). Predictor variables were pre-screened for multicollinearity; if a pair of variables had a Pearson *r* value greater than 0.85, one variable was eliminated based on *a priori* expectations. Predictor variables with a tolerance value < 0.35 or a Variance inflation factor (VIF) >3 were also eliminated, as was one influential case identified by having a Cooks distance >1 (Field 2013). All results are expressed as mean ± standard deviation unless otherwise stated with statistical significance accepted at $P \leq 0.05$.

4.1.3 Results

4.1.3.1 Participant characteristics and nutritional intake

Participant characteristics and their nutritional intake are reported in Table 4-1 and).

Table 4-2, respectively. As expected from differences between the sexes in body size, men were significantly taller, heavier, had a larger waist circumference, greater BMI, lower body fat (%), less FM, more FFM, a higher ratio of abdominal to lower body fat mass and a greater $\dot{V}O_2$max. Similarly, men's total absolute energy intake and energy expenditure was higher than the women's, with men reporting a greater absolute intake of protein, fat and CHO, as well as alcohol. For men, there was a greater % contribution to total energy intake from protein and alcohol and less from CHO than women, with no significant difference in fat intake. There were no differences between the sexes in age, SRPAL or energy balance.

4.1.3.2 Substrate oxidation

The MFO was 0.55 ± 0.19 (range: 0.19-1.13) g/min or 10.8 ± 3.2 (range: 3.5-20.7) mg/kg FFM/min and occurred at 60 ± 16 (range: 19-92) % $\dot{V}O_2$max (FatMax). The absolute MFO (g/min) was greater in men (0.62 ± 0.19 [0.21-1.13] g/min) than in women (0.48 ± 0.15 [0.19-0.99] g/min) (P <0.0001), whereas it was lower in men when expressed relative to FFM (men, 10.3 ± 3.1 [3.5-19.9]; women, 11.2 ± 3.3 [4.6-20.7] mg/kg FFM/min) (P<0.05). There was no statistically reliable difference (P = 0.09) in the exercise intensity at which MFO occurred (i.e., FatMax) between men (58.7 ± 15.9% [22.9-91.4]) and women (61.8 ± 15.7% [19.3-92.3] $\dot{V}O_2$max). The percentage contribution of FAT and CHO-OX to energy expenditure for each sex is displayed in Figure 4-2. Proportionally, fat contributed as the main fuel source at low exercise intensities until approximately 50% $\dot{V}O_2$max after which CHO became the dominant source of energy. The relative contributions of fat and carbohydrate to energy expenditure were higher and lower, respectively, in women as compared to men. A similar

pattern for sex-differences in FAT and CHO-OX were observed when oxidation rates were expressed relative to FFM (Figure 4-3).

Table 4-2 Macronutrient intake and related parameters

Variable	All Subjects (*n*=305)	Men (*n*=150)	Women (*n*=155)
Energy Expenditure (kcal/day)	2568 ± 445	2912 ± 348***	2236 ± 219
Energy Balance (kcal/day)	75 ±553	103 ± 615	47 ± 486
Energy Intake (kcal/day)	2608 ± 738	3001 ± 719***	2227 ± 527
% Fat to Energy Intake	34.3 ± 6.9	34.2 ± 7.0	34.4 ± 6.9
% Protein to Energy Intake	17.3 ± 5.8	19.1 ± 7.0***	15.5 ± 3.6
% Carbohydrate to Energy Intake	45.4 ± 9.3	43.1 ± 9.7***	47.5 ± 8.3
% Alcohol to Energy Intake[2]	2.6 ± 4.7	3.3 ± 4.9***	2.1 ± 4.3
Total Fat (g/day)	99 ± 34	113 ± 33***	86 ± 28
Total Protein (g/day)	113 ± 49	141 ± 52***	86 ± 26
Total Carbohydrate (g/day)	313 ± 104	347 ± 114***	281 ± 82
Total Alcohol (g/day)[2]	11 ± 21	15 ± 25***	7 ± 15

[1] All values are Mean ± SD; *** Significantly (*P* <0.001) different from Women (unless stated: independent *t* test, [2] Mann-Whitney U test).

4.1.3.3 Determinants of MFO

Analysis employed a two-step hierarchical regression with fitness and body size related variables expected to be associated with MFO entered on step 1 and macronutrient intake variables on step 2. The macronutrient intake values were entered in absolute terms (g/day),

as opposed to relative contribution to Energy Intake (%) (EI). When expressed as a % of EI, the macronutrients were inevitably linked to each other by the common divisor of % EI used in their derivation. If macronutrients were expressed as % EI, it would be impossible to determine their independent contribution to the variability in MFO. The simple bivariate correlations between energy balance or A/LB FM and MFO were not significant and were therefore not entered in the hierarchical regression (energy balance: r = 0.08, P = 0.18, r = 0.09, P = 0.11; A/LB FM: r = -0.05, P = 0.35, r = -0.08, P = 0.15; for MFO expressed as g/min or mg/kg FFM/min, respectively).

The results of hierarchical regression analysis with the dependent variable of absolute MFO (g/min) are summarized in Table 4-3. $\dot{V}O_2$max, SRPAL and sex accounted for 43.5% of the variability in MFO, with no significant contribution from either FFM or FM. The step 2 analysis improved the amount of variability explained by 2.7%, and showed CHO and fat intake to be significant independent predictors of MFO. The relative magnitude of the standardized coefficients indicates the magnitude of their independent contribution. Positive standardized coefficients for $\dot{V}O_2$max, SRPAL, and fat intake indicate that these variables are positive predictors of MFO (i.e., an independent increase in any of these variables corresponds to a greater MFO). The negative standardized coefficient for CHO intake indicates a reduction in MFO following an increase in this variable, with sex also having a negative association by virtue of statistical coding (men, 1; women, 2).

A similar two-step hierarchical regression procedure was used for MFO relative to FFM (mg/kg FFM/min) with one exception. FFM could not be included in the analysis summarized

in Table 4-4, because of its contribution to the dependent variable**.** $\dot{V}O_2$max, SRPAL, sex and FM all contributed, and together accounted for 17.4% of the variability in MFO. All three macronutrients made significant independent contributions to the step 2 analysis, explaining a further 3.1% of variability. $\dot{V}O_2$max, SRPAL, sex (coding: men 1, women, 2) and fat intake had positive relationships with MFO, whereas the associations for FM, CHO and protein intake were negative.

Table 4-3 Hierarchical Multiple Linear Regression for absolute Maximal Fat Oxidation (g/min) (n=305)

		Absolute MFO (g/min) (*n=3*05)			
Model	Independent Variables	Unstandardized Coefficients	Standardized Coefficients	R^2 (95% CI) [3]	Adjusted R^2
		Beta (95% CI)	Beta		
1				0.444 [***1] (0.368–0.525)	0.435
	$\dot{V}O_2$max (L/min)	0.089 (0.063 – 0.155)	0.477 ***		
	SRPAL[1]	0.037 (0.018 – 0.055)	0.196 ***		
	Sex (M = 1, W = 2)	-0.005 (-0.175 – -0.112)	-0.385 ***		
	FM (kg)	-0.017 (-0.022 – 0.013)	-0.024		
	FFM (kg)	-0.143 (-0.041 – 0.008)	-0.091		
2				0.476 [**1] (0.409–0.570)	0.462
	$\dot{V}O_2$max (L/min)	0.098 (0.072 – 0.124)	0.527 ***		
	SRPAL[1]	0.044 (0.025 – 0.064)	0.238 ***		
	Sex (M = 1, W = 2)	-0.143 (-0.174 – -0.113)	-0.385 ***		
	FM (kg)	-0.005 (-0.022 – 0.012)	-0.025		
	FFM (kg)	-0.021 (-0.046 – 0.003)	-0.115 †		
	Protein intake (g)	-0.009 (-0.027 – 0.010)	-0.046		
	Carbohydrate Intake (g)	-0.033(-0.051 – -0.015)	-0.178 ***		
	Fat Intake (g)	0.027 (0.009 – 0.044)	0.144 **		

[1]Significant change in R^2. Significance of *P* value *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. † $P < 0.10$. [3] Bootstrapped 95% CI. FFM, Fat Free Mass; MFO, Maximal Fat Oxidation; SRPAL, Self-Reported Physical Activity Level; $\dot{V}O_2$max, maximal oxygen uptake.

Table 4-4 Hierarchical Multiple Linear Regression for MFO relative to FFM (mg/kg FFM/min)

	Relative MFO (mg/kg FFM/min) (*n*=305)				
Model	Independent Variables	Unstandardized coefficients	Standardized coefficients	R^2 (95% CI)[3]	Adjusted R^2
		Beta (95% CI)	Beta		
1				0.185 ***[1] (0.116 – 0.276)	0.174
	$\dot{V}O_2$max (L/min)	0.459 (0.084 – 0.834)	0.143 *		
	SRPAL[1]	0.838 (0.450 – 1.226)	0.261 ***		
	Sex (M = 1, W = 2)	0.873 (0.214 – 1.532)	0.136 **		
	FM (kg)	-0.459 (-0.802 – -0.117)	-0.143 **		
2				0.223 **[1] (0.156 – 0.327)	0.205
	$\dot{V}O_2$max (L/min)	0.630 (0.241 – 1.020)	0.196 **		
	SRPAL[1]	1.025 (0.624 – 1.426)	0.319 ***		
	Sex (M = 1, W = 2)	0.873 (0.226 – 1.519)	0.136 **		
	FM (kg)	-0.509 (-0.848 – -0.170)	-0.158 **		
	Protein intake (g)	-0.408 (-0.786 – -0.030)	-0.127 *		
	Carbohydrate Intake (g)	-0.609 (-0.990 – -0.228)	-0.189 **		
	Fat Intake (g)	0.472 (0.102 – 0.843)	0.147 *		

[1]Significant change in R^2 . Significance of *P* value *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. † $P < 0.10$.[3] Bootstrapped 95% CI intervals. FFM, Fat Free Mass; MFO, Maximal Fat Oxidation; SRPAL, Self-Reported Physical Activity Level; $\dot{V}O_2$max, maximal oxygen uptake

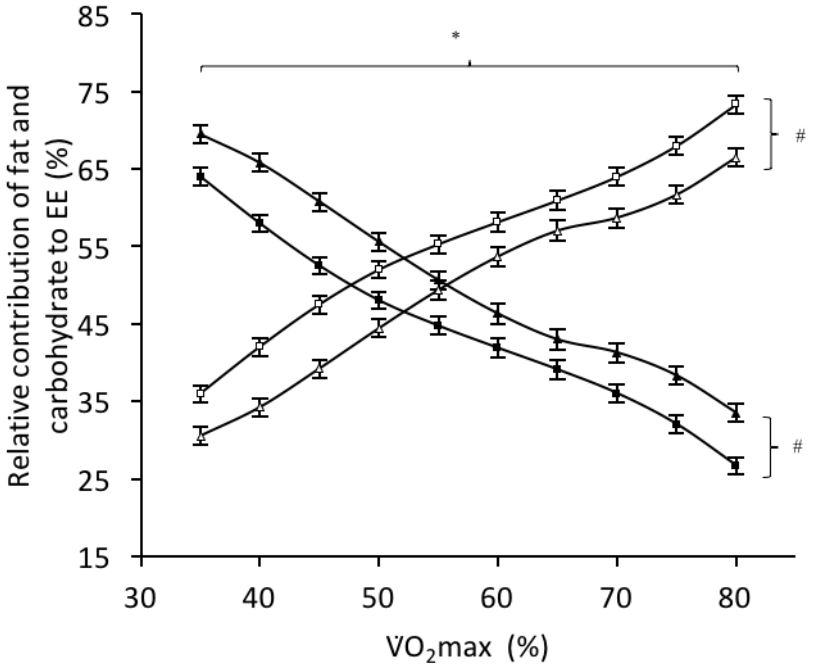

Figure 4-2 Sex differences in the proportional (% total energy expenditure) contribution of fat and carbohydrate oxidation rates during incremental exercise between 35-80% $\dot{V}O_2$max (men *n* =145, women *n* = 135)

Women fat oxidation ▲; women carbohydrate oxidation △, men fat oxidation ■; men carbohydrate oxidation □. # $P < 0.01$ for main effect of sex, * $P < 0.001$ for main effect of exercise intensity and $P < 0.05$ for a sex by exercise intensity interaction using a mixed ANOVA. EE, energy expenditure; $\dot{V}O_2$max, maximal oxygen uptake

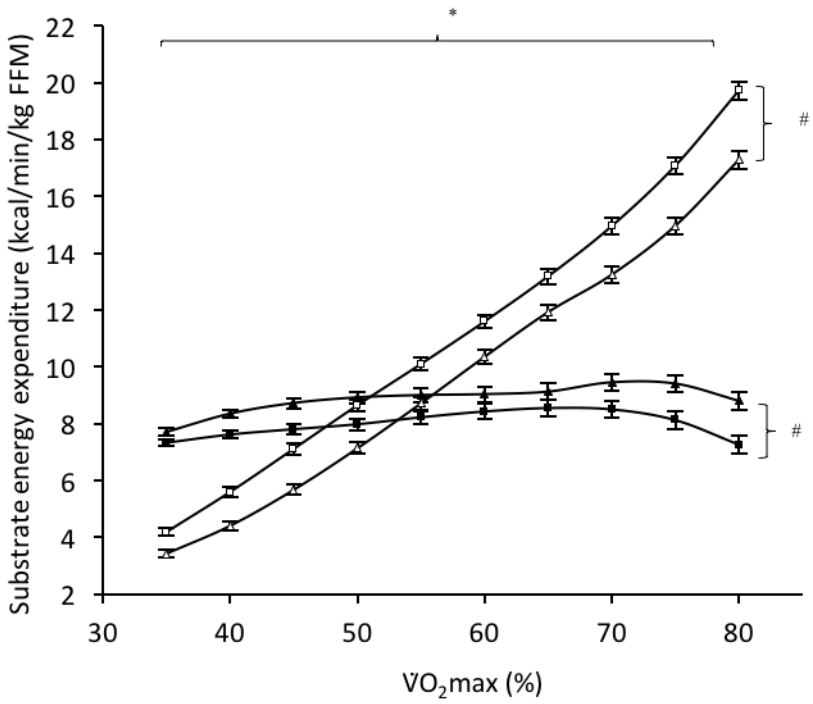

Figure 4-3 Sex differences in substrate energy expenditure relative to FFM (kcal/min), during incremental exercise between 35-80% $\dot{V}O_2$max (men n=145, women n = 135)

Values are means, error bars represent SEM. Women fat oxidation ▲; women carbohydrate oxidation △, men fat oxidation ■; men carbohydrate oxidation □.

# $P < 0.01$ for main effect of sex, * $P < 0.001$ for main effect of exercise intensity and $P < 0.05$ for a sex by exercise intensity interaction using a mixed ANOVA.

4.1.4 Discussion

The present study tested the association of recent self-selected dietary intake on the inter-individual variability in MFO during exercise in healthy young men and women. The total amount of variability explained was 46.2% and 20.5% of absolute (g/min) and relative MFO

(mg/kg FFM/min), respectively, with diet accounting for ~3% of this. This study also demonstrated that biological sex is a determinant of MFO, independent of other important predictors on fat oxidation. While men had a higher absolute MFO (g/min) than women, the reverse was true when MFO was expressed relative to FFM.

We confirm that substantial inter-individual variability exists in MFO, ranging by almost 6-fold (0.19-1.13 g/min; 3.5-20.7 mg/kg FFM/min). The non-diet related variables in the analyses explained 43.5% and 17.4% of the variability in absolute (g/min) and relative MFO (mg/kg FFM/min), respectively. These amounts are greater than previously reported using similar predictor variables (35% and 12%, respectively) (Venables, Achten, and Jeukendrup 2005). Here, participants were fully familiarized with testing procedures (vs. no familiarization) and MFO was determined during exercise performed in the overnight-fasted state (vs. minimum 4 hrs. postprandial). A detailed diary was used to assess physical activity rather than a brief questionnaire. Body composition was assessed with DXA scanning rather than the skin-fold technique. These methodological improvements, affording greater precision and reliability of measurement, would account for the greater explained variance. In addition, the main objective of the study, dietary intake, explained a further ~3% of the inter-individual variability in MFO during exercise.

CHO and fat intake were the most consistent dietary determinants of MFO, explaining variability in MFO expressed in both absolute and relative terms, whereas protein intake was only related to the latter. Higher CHO intake was associated with reduced MFO, echoing

controlled short-term dietary manipulation studies where marked isoenergetic increases in dietary CHO decreases FAT-OX (Bergstrom et al. 1967; Lambert et al. 1994; Helge, Wulff, and Kiens 1998). This is likely to reflect a direct influence of CHO intake on its subsequent availability for oxidation during exercise (Bergstrom et al. 1967) and/or the related anti-lipolytic effect of insulin (Horowitz et al. 1997). Increasing glycolytic flux during exercise (e.g., with high exercise intensities or increased CHO provision) can also directly down-regulate mitochondrial long-chain FAT-OX (Sidossis et al. 1997; Coyle et al. 1997). The positive association of fat intake with MFO is also consistent with previous studies where dietary fat intake was associated with FAT-OX (Goedecke et al. 2000; Cameron-Smith et al. 2003; Volek et al. 2016; Helge et al. 2001). Fat intake has been suggested to influence FAT-OX through several mechanisms including greater plasma and intramuscular lipid availability (Zderic et al. 2004; Johnson et al. 2003), greater expression key proteins involved in cellular fatty acid uptake (Helge et al. 2001; Cameron-Smith et al. 2003; Goedecke et al. 1999) and beta oxidation (Helge and Kiens 1997; Cameron-Smith et al. 2003) and reciprocal down regulation of enzymes (e.g. pyruvate dehydrogenase) involved carbohydrate oxidation (Stellingwerff et al. 2006; Peters 2003; Putman et al. 1993). Collectively, the present study clarifies an independent role of both carbohydrate and fat intake in the modulation of FAT-OX, even within the context of typical dietary patterns.

Unexpectedly, protein intake was associated with reduced FAT-OX when MFO was expressed relative to FFM; the reason for this effect is unclear. I did not include measures of urinary nitrogen excretion, which may have affected the accuracy of estimates of substrate oxidation although we think this explanation unlikely, as a similar independent association of

protein intake on absolute MFO was not seen. Contrary to suggestions that negative energy balance would increase FAT-OX (Braun and Brooks 2008), but consistent with Rosenkilde *et al* (Rosenkilde et al. 2010), we found no relationship between energy balance and MFO. The potential limitations of estimating energy intake and expenditure using self-report in free-living individuals are well documented (Schoeller 1995; Irwin, Ainsworth, and Conway 2001). Nonetheless, detailed activity diaries coupled with weighed food intake should provide relatively accurate data. Further, the direction of relationships for both CHO and fat intake with MFO matched prior expectations. Therefore, the data suggest short-term (i.e., 4 - day) energy balance is not a substantial contributor to inter-individual variability in MFO.

The variability in MFO attributed to diet could appear modest (~3%) and thus the importance of this observation warrants consideration. Clearly, the large effects of aerobic fitness and physical activity level on MFO underline their importance when targeting FAT-OX for metabolic health. Nonetheless, we studied free-living participants under minimal dietary constraints. Variation between individuals occurred under conditions that mimicked their daily lives. Dietary intake emerged as an independent predictor of MFO in addition to factors previously shown to exert large effects. Thompson *et al* (Thompson et al. 2012) suggested a ~5% increase in FAT-OX whilst physically active could make important contributions to the maintenance of daily fat balance. This is supported by our recent linking of the capacity for FAT-OX during exercise with 24 hour FAT-OX (Robinson et al. 2015). The findings here, in a large, diverse healthy population, suggest dietary macronutrient manipulation could exert modest yet meaningful effects on FAT-OX during physical activity applicable to real-world settings.

A key strength of our analysis is the minimization of the influence of typically observed sex-related differences in the measured independent variables. This allows us to make conclusions not previously possible (Venables, Achten, and Jeukendrup 2005) and state with greater certainty that $\dot{V}O_2$max, SRPAL and biological sex (i.e., women vs. men) are positive determinants of MFO (Venables, Achten, and Jeukendrup 2005). Our data showing a greater (~10%) FAT-OX in women across a range of exercise intensities (Figure 4-2) is confirmatory (Tarnopolsky et al. 1990; Cheneviere et al. 2011; Venables, Achten, and Jeukendrup 2005) but the independent effect of biological sex on MFO has not to our knowledge previously been reported. It is noteworthy that higher FAT-OX was observed in women despite the men exhibiting a small but significantly higher aerobic capacity ($\dot{V}O_2$max (ml/kg FFM/min; Table 4-1). This further highlights the independent role of sex-related differences as determinants of fat oxidation during exercise. Other physiological differences unaccounted for in this study, such as ovarian hormones or intra-muscular substrate availability, are possible candidates for the enhanced FAT-OX in women (see section 2.3.5, and (Tarnopolsky et al. 1990; Lundsgaard and Kiens 2014)).

Appropriate statistical adjustment for baseline sex-differences in aerobic fitness, body-size related variables and diet, and the robust assessment of body composition using DXA also clarify the role of body composition as a determinant of MFO. We observed no effect of FFM on MFO, contradicting Venables *et al* (Venables, Achten, and Jeukendrup 2005). It has recently been suggested that the location of FM is also related to MFO (Isacco et al. 2013),

however, using our much larger cohort, we see no relationship between body fat distribution and MFO. Nonetheless we do show total FM to be a negative determinant of MFO expressed relative to FFM. Our recent work shows positive relationships between MFO and both overnight-fasted and total daily FAT-OX (Robinson et al. 2015), both of which are linked with obesity risk (Zurlo et al. 1990; Shook et al. 2015). Thus, a low MFO could be a factor in FM accumulation through reduced FAT-OX whilst physically active, or could reflect a more generalized reduced capacity to oxidize fat.

In summary, self-selected dietary intake was shown to have a modest association with the maximal rate of FAT-OX during exercise. Biological sex was also an independent predictor, with women showing a higher maximal fat oxidation relative to FFM than men. Collectively, the study highlights the importance of modifiable lifestyle factors such as fitness, physical activity and diet in determining FAT-OX during physical activity at intensities consistent with current public health recommendations, with implications for the optimization of metabolic health, body mass and composition.

4.2 Potential association of the menstrual cycle and hormonal contraceptive use on the maximal rate of fat oxidation during exercise

The following section describes and then discusses potential differences in substrate oxidation in the cohort of women from section 4.1 when classified by the phase of the menstrual cycle and/or hormonal contraceptive use. As outlined within the methods section of Chapter 4.1, the data relating to both the phase of the menstrual cycle and hormone contraceptive use were obtained qualitatively through a questionnaire (see Appendix 7.4). Thus, the absence of directly measured blood sex-steroid concentrations to objectively verify menstrual cycle phase should be considered when interpreting the analysis presented. A brief background to each topic shall be given with a description of the data analysis undertaken for each topic and the results followed by a collective discussion.

4.2.1 Menstrual Cycle

In regularly menstruating women, the ovarian hormones; oestrogen and progesterone in addition to other gonadotropic hormones follow a well-defined pattern over the course of a full menstrual cycle (Speroff and Van De Wiele 1971) (see Figure 4-4). The menstrual cycle can be divided into three general phases based upon events in the follicles of the ovary and the resultant changes in hormonal milieu; the follicular phase, ovulation and the luteal phase. As seen in Figure 4-4, the plasma concentrations of the ovarian and gonadotropic hormones fluctuate markedly throughout the cycle, with oestrogen ranging from 10 –

300pg/ml. Oestrogen has been shown to influence metabolism during exercise. For instance, ovariectomised rats supplemented with oestrogen oxidise less carbohydrate, sparing both hepatic and muscle glycogen, with a concomitant greater lipid availability and oxidation (Kendrick and Ellis 1991; Rooney et al. 1993). Indeed <1 week oestrogen supplementation in men, results in a pattern of fuel use on a whole body level much more typical of women; with reductions in carbohydrate utilisation and greater lipid oxidation (Hamadeh, Devries, and Tarnopolsky 2005; Ruby et al. 1997; Carter et al. 2001; Devries et al. 2005)

The widely divergent hormonal profiles throughout a typical menstrual cycle, in particular that of oestrogen, is often cited as the driving force behind differences in metabolism and substrate oxidation reported between the phases. Greater rates of lipid oxidation have been reported during the luteal phase (a sustained elevation of oestrogen) compared to the follicular (Hackney 1999; Zderic, Coggan, and Ruby 2001; Campbell, Angus, and Febbraio 2001) and this is associated with greater oxidation of circulating NEFAs (Zderic, Coggan, and Ruby 2001; Hackney, McCracken-Compton, and Ainsworth 1994). Higher carbohydrate oxidation often observed during the follicular phase is associated with a greater systemic glucose turnover when fasted or when the exercise intensity is greater than 55% $\dot{V}O_2$max (Devries et al. 2006; Campbell, Angus, and Febbraio 2001; Ruby et al. 1997). The effects on muscle glycogen storage are less clear, with greater repletion seen in the luteal phase (Nicklas, Hackney, and Sharp 1989) or no differences in storage but a greater rate of utilisation seen in the follicular phase than luteal following 90mins cycling at 65% $\dot{V}O_2$max during exercise (Devries et al. 2006). However, these differences in substrate storage,

oxidation and metabolism between the menstrual cycle phase have not always been reported (Galliven et al. 1997; Vaiksaar et al. 2011; Isacco et al. 2012; Kanaley et al. 1992) even when under strict dietary control and testing across three distinct hormonal phases of the cycle (Horton et al. 2002).

Thus, there is clearly some discrepancy in the literature regarding substrate oxidation during exercise during the different phase of the menstrual cycle, some of which may be explained by limitations in statistical power with many studies using small (often only *n* = 5-10) samples sizes. It was considered pertinent therefore to explore further in our relatively large cohort of women to ascertain if MFO showed any evidence of variation by estimated menstrual cycle phase.

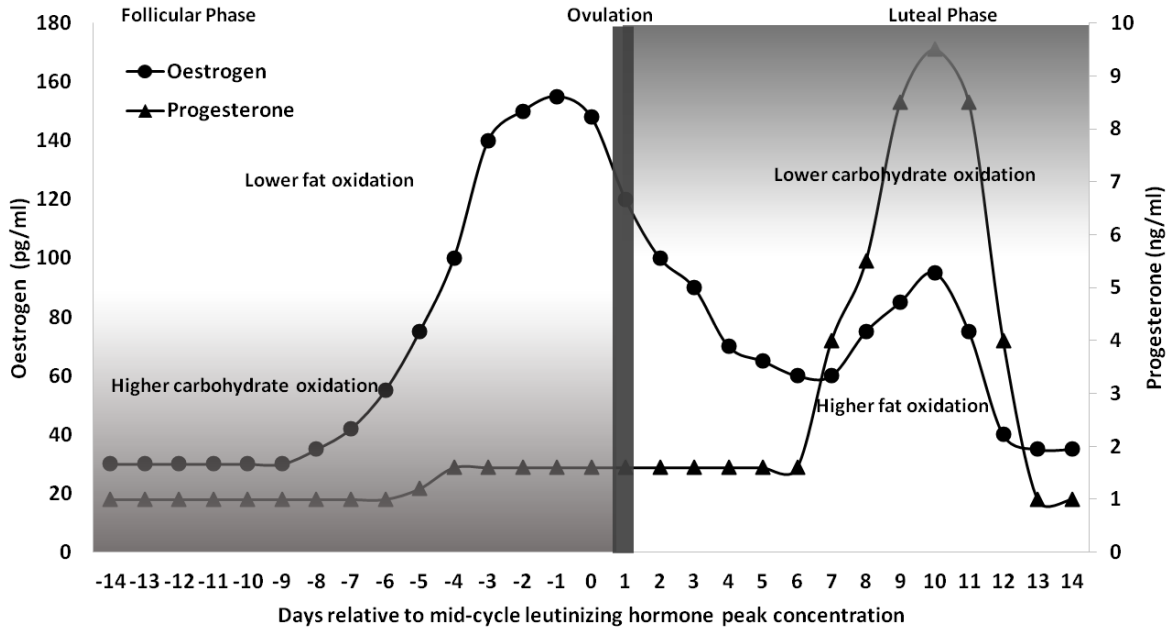

Figure 4-4 Typical monthly ovarian hormonal profile.

Markers represent typical ovarian hormone concentration over an average 28 day cycle. The background colour represents the hypothetical relative proportion (%) of carbohydrate (grey area) and fat (white area) oxidation to total energy expenditure during moderate intensity exercise.

4.2.1.1 Methods

Using a calendar based approach to estimate the phase of the menstrual cycle that testing occurred in, the women were categorized into three groups; Follicular (FOL), Luteal (LUT) or Amenorrhoeic (contraceptive users only). Those tested within the first half of their average menstrual cycle (i.e. within 0-14days of a 28-day cycle) were classified as FOL (*n* = 57), whereas those in the second half (days 15-28) were LUT (*n* = 82) with contraceptive induced amenorrhea (*n* = 16) a 3rd group. This level of grouping combined both contraceptive and

non-contraceptive users (see row “Total” in Table 4-5), which also includes a more complete breakdown by use of different contraceptive type).

Table 4-5. Distribution of groups split by phase of the menstrual cycle and type of hormonal contraception used

		Phase of Menstrual Cycle			
		Luteal	Follicular	Amenorrhoeic	Total
Type of Contraception	E+P	28	17	1	46
	PROGEST	2	2	15	19
	REG	52	38	0	90
	Total	82	57	16	155

E+P; oestrogen and Progesterone combined contraceptive, PROGEST; Progesterone only contraceptive, REG; Regularly menstruating no contraceptive use

4.2.1.2 Statistics

The three groups were normally distributed as assessed by distribution and QQ plots with non-significant Kolmogorov-Smirnov tests. Levene's test for equality of variance was met for the absolute MFO but was violated for the MFO relative to FFM, therefore, differences between groups for absolute MFO were explored using a one-way ANOVA and Welchs ANOVA for MFO (mg/kg FFM/min). Hochbergs GT2 post hoc tests were carried out to identify where the groups differed in absolute MFO and relative to FFM due to differences in group sizes. Data are presented as means ± SD and the range in parenthesis, with statistical significance accepted at ($p \leq 0.05$).

4.2.1.3 Results

The absolute MFO (g/min) was significantly greater in the amenorrhoeic women (0.59 ± 19) than both those in either the luteal (0.49 ± 0.14) ($p<0.05$) or follicular phase (0.43 ± 0.13) ($p<0.01$) of the menstrual cycle, with a trend ($p=0.063$) for the follicular phase to be lower than the luteal. Similarly, when expressed relative to FFM the women that were amenorrhoeic during testing had a significantly higher MFO (13.6 ± 4.3 mg/kg FFM/min) than both the follicular (10.2 ± 2.6) ($p<0.01$) and luteal groups (11.4 ± 3.2 mg/kg FFM/min) ($p<0.05$), with a trend ($p=0.08$) for the follicular phase to be lower than those in the luteal.

Table 4-6. The MFO (g/min) of women split into groups by phase of the menstrual cycle and type of hormonal contraception used

		Phase of Menstrual Cycle			
		Luteal	Follicular	Amenorrhoeic	Total
Type of Contraception	E+P	0.54 ± 0.14 (0.31-0.84)[4]	0.49 ± 0.13 (0.29-0.73)	0.80 (n=1)	0.53 ± 0.14 (0.29-0.84)
	PROGEST	0.51 ± 0.21 (0.36-0.66)	0.42 ± 0.16 (0.31-0.53)	0.58 ± 0.19 (0.31-0.99)[4,5]	0.55 ± 0.19 (0.31-0.99)
	REG	0.46 ± 0.13 (0.19-0.82)[5]	0.40 ± 0.12 (0.20-0.70)[4]	NA	0.43 ± 0.13 (0.19-0.82)[3]
	Total	0.49 ± 0.14 0.19-0.84)	0.43 ± 0.13 (0.20-0.73)[2]	0.59 ± 19 (0.31-0.99)[1]	0.59 ± 19 (0.31-0.99)[1]

E+P; oestrogen and Progesterone combined contraceptive, PROGEST; Progesterone only contraceptive, REG; Regularly menstruating no contraceptive use. Data is mean ± SD, range in parenthesis. [1] Amenorrhoeic significantly different ($P< 0.01$) than Luteal and Follicular ($P< 0.05$). [2] Trend ($P=0.06$) for follicular to be different to Luteal. [3] REG significantly ($P <0.01$) different than E+P and PROGEST. [4] REG-FOL significantly ($P <0.01$) different to PROGEST-A and E+P-LUT. [5] Trend ($P =0.06$) for PROGEST-A different to REG-LUT

Table 4-7. The MFO (mg/kg FFM/min) of women split into groups by phase of the menstrual cycle and type of hormonal contraception used

		Phase of Menstrual Cycle			
		Luteal	Follicular	Amenorrhoeic	Total
Type of Contraception	E+P	12.5 ± 3.2 (7.3-19.7)	11.6 ± 2.3 (8.6-15.8)	19.3 (*n*=1)	12.3 ± 3.0 (7.3-19.7)
	PROGEST	11.8 ± 3.6 (9.3-14.3)	9.6 ± 1.0 (8.9-10.3)	13.2 ± 4.1 (7.3-20.7)	12.7 ± 3.9 (7.3-20.7)
	REG	10.8 ± 3.2 (5.4-19.6)	9.6 ± 2.6 (4.6-16.8)[4]	NA	10.3 ± 3.0 (4.6-19.6)[3]
	Total	11.4 ± 3.2 (5.4-19.7)	10.2 ± 2.6 (4.6-16.8)[2]	13.6 ± 4.3 (7.3-20.7)[1]	

E+P; oestrogen and Progesterone combined contraceptive, PROGEST; Progesterone only contraceptive, REG; Regularly menstruating no contraceptive use. Data is mean ± SD, range in parenthesis. [1] Amenorrhoeic significantly different (P< 0.01) than Luteal and Follicular (P< 0.05). [2] Trend (P=0.08) for follicular to be different to Luteal. [3] REG significantly different than E+P (P <0.01) and PROGEST (P <0.05). [4] REG-FOL significantly (P <0.01) different to PROGEST-A and E+P-LUT.

4.2.2 Hormonal contraceptive use

There are many different types of hormonal contraceptives currently prescribed in the UK. They can be broadly categorized as either a combined contraceptive, containing a synthetic oestrogen and progestogen (which can vary in proportions over a monthly cycle if bi or tri-phasic), or as a progestogen only type of contraceptive (PROGEST). The effect of the exogenous oestrogen component of the E+P contraceptives on substrate metabolism has

been described in section 2.3.5.1.1. The progestogen component has the primary function of suppressing the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), inhibiting follicular development and preventing the normal mid-cycle surge in oestrogen and LH, (Ruan, Seeger, and Mueck 2012) accumulating in the prevention of ovulation and lower sustained oestrogen levels.

In contrast to the administration of oestrogen which has demonstrated increased rates of lipid utilisation during exercise (Ruby et al. 1997), with greater activity of key enzymes involved in mitochondrial lipid transport and oxidation (Campbell and Febbraio 2001; Fu et al. 2009), the evidence for the independent effect of PROGEST is less clear. Rodent studies show a reduction in the potential for lipid oxidation following PROGEST administration. For instance, Campbell & Febbraio (Campbell and Febbraio 2001) subjected ovariectomised rats to 15days of PROGEST only (to correspond to normal physiological doses) and reported ~20% reduction in the maximal activity of CPT1 and B-HAD compared to control rats. Unfortunately, no measures of lipid oxidation were made but with a potentially lower LCFA transport capacity into the mitochondria, and lower B-HAD activity, a reduction in lipid oxidation would be likely. Furthermore, the addition of oestrogen (to correspond to normal physiological doses) to PROGEST could not recover the attenuation in activity of CPT1. Oestrogen alone however, or in a supra-physiological dose combined with PROGEST increased CPT1 activity 14 and 19% respectively with a similar result for B-HAD, suggesting oestrogen can overcome the deleterious effects of PROGEST when the ratio of oestrogen: progesterone is >2 (D'Eon et al. 2002). Oestrogen administration compared to progesterone also results in greater increases in PPAR-alpha mRNA and protein abundance, and increases

the glycolytic down-regulatory enzyme PDK4 gene expression by 23-fold compared to a 12-fold increase with progesterone, suggesting a blunting of the response by progesterone (Campbell et al. 2003). With these enzymes and transcription factors important in either up regulating lipid oxidation or down regulating the glycolytic pathway respectively.

The majority of literature concerning the metabolic effects of hormonal contraceptives on substrate metabolism during exercise are based on investigations using E+P contraceptives, using 2nd or 3rd generation synthetic hormones. The current (4th generation) contraceptives use different oestrogens and progestins that are typically lower in androgenocity and of lower hormonal doses due to increased potency (Burrows and Peters 2007). For example, ethinyl estradiol is over 100 fold more effective regarding its effect on serum SHBG than older oestrogen preparations like piperazine oestrone sulphate (Grow 2002), and so the applicability of investigations using older forms of contraceptives although provide useful insights, should be viewed with caution before extrapolating to findings using newer preparations.

Whilst it is well established that oestrogen administration reduces glucose Ra and Rd (Ruby et al. 1997; Devries et al. 2005; Carter et al. 2001) and favours lipid oxidation during exercise, the effect of E+P contraceptives on substrate oxidation and metabolism is less clear. Generally, no effect on whole body lipid or carbohydrate oxidation has been reported when comparing triphasic E+P contraceptive (Casazza et al. 2004; Tremblay et al. 2010; Jacobs et al. 2005; Suh et al. 2003; Lebrun et al. 2003). Whereas the literature on

monophasic E+P is less clear, with either no differences in whole body substrate oxidation seen (Isacco et al. 2012) or a lowering of carbohydrate oxidation compared to non-users (Bonen, Haynes, and Graham 1991; Bemben et al. 1992; McNeill and Mozingo 1981). Despite the discrepancies regarding whole body substrate oxidation rates, most studies still report other metabolic changes following E+P use indicative of altered lipid metabolism.

For instance, in a longitudinal study comparing values taken 4 months prior to tri-phasic contraceptive use, Casazza *et al* (Casazza et al. 2004) found E+P to increase TAG mobilisation and whole body lipolysis with a ~20% greater Ra of glycerol during exercise at 45 and 65% $\dot{V}O_2$peak. Using the same contraceptive, the same research group also reported an increase in lipolytic rate, no change in plasma NEFA oxidation and an apparent increase in the rate of NEFA re-esterification during exercise (Casazza et al. 2004; Jacobs et al. 2005). These greater rates of lipolysis and an 11% fall in the Ra, Rd and MCR of glucose (Suh et al. 2003) all occurred despite exercise testing commencing 3hrs post-prandially, which would be expected to lessen any observed effect. From the work of Casazza and colleagues (Casazza et al. 2004; Jacobs et al. 2005) they concluded that the OCs they tested was second only to energy flux (exercise intensity) in effecting triglyceride mobilization, having a greater impact than recent CHO intake and menstrual cycle phase. Mono-phasic E+P contraceptives appear to demonstrate similar effects, with greater plasma NEFA and TAG mobilisation, with reductions also seen in glycolysis and systemic glucose availability (McNeill and Mozingo 1981; Bonen, Haynes, and Graham 1991; Carter et al. 2001; Devries et al. 2005).

Thus, it appears the exogenous oestrogen in E+P contraceptives, enhance systemic lipid availability whilst reducing carbohydrate availability, even if it does not always translate to an impact upon rates of whole body substrate oxidation. Furthermore, it could be hypothesized that the PROGEST contraceptive group, with its oestrogen suppressive or potentially antagonistic effect, might be associated with a lower MFO compared to E+P or REG.

4.2.2.1 Methods

Of the 155 women who participated in the study, 90 were not using any form of contraceptive, the remaining 65 used a total of 11 differe*n*t types of hormonal contraceptive: with the combined (E+P) contraceptives; Femodene (ethinylestradiol and gestodene) (*n*=3); Microgynon 30 (ethinylestradiol and levonorgestrel) (*n*=22); Marvelon (ethinylestradiol and desogestrel) (*n*=3); Yasmin (ethinylestradiol and drospirenone) (*n*=11); Gederal (30/150 ethinylestradiol and desogestrel) (*n*=1); Cilest (ethinylestradiol and norgestimate) (*n*=4); Mercilon (ethinylestradiol and desogestrel) (*n*=1); Logynon (ethinylestradiol and levonorgestrel) (*n*=1); and the progesterone only forms of contraceptives (PROGEST); Nexplanon implant (etonogestrel) (*n*=4); Mireina Coil (levonorgestrel) (*n*=4); Depo-Provera injection (medroxyprogesterone acetate) (*n*=5); Cerazette (desogestrel) (*n*=5); Implanon implant (etonogestrel) (*n*=1). These were later grouped together as either PROGEST (*n*=19) or E+P (*n*=46) and were compared to those who were regularly menstruating and not using contraceptive (*n*=90) (REG).

4.2.2.2 Statistical analysis

For the absolute MFO (g/min) the groups were normally distributed as assessed by distribution and QQ plots with non-significant Shapiro-Wilk tests, with equal variances between groups assessed by Levene's test for equality of variance. When the MFO was expressed relative to FFM the data was not normally distributed, however, a One-way ANOVA is deemed to be robust against this violation and so with the equal variances between groups (non-significant Levene's test for equality of variance) it was used to determine differences between the groups with Hochbergs GT2 post hoc tests to identify where the groups differed. Values for both the absolute MFO (g/min) and relative to FFM (mg/kg FFM/min), are expressed as means $\pm$ SD with statistical significance accepted at $p \leq 0.05$.

4.2.2.3 Results

The absolute MFO (g/min) was significantly ($p<0.01$) lower in the REG group (0.43 ± 1.3) compared to both the PROGEST (0.55 ± 0.19) and the E+P (0.53 ± 0.14) with no significant differences between the type of contraception. Similarly when the MFO was expressed relative to FFM the REG group had a significantly lower MFO (10.3 ± 3.0 mg/kg FFM/min) than both the PROGEST (12.7 ± 3.9 mg/kg FFM/min) ($p<0.01$) and the E+P (12.3 ± 3.0 mg/kg FFM/min) ($p<0.01$), with no significant difference between the PROGEST and E+P.

4.2.3 Type of contraceptive and menstrual cycle phase interaction

To investigate if there was an interaction effect between the phase of the menstrual cycle and contraceptive use with MFO the women were split into 8 groups. Those that were regularly menstruating with no contraceptive use were either; follicular (REG-FOL) or luteal (REG-LUT), with those using a combined type contraceptive; in the follicular phase (E+P+FOL), luteal (E+P+LUT) or amenorrhoeic (E+P+A); those using a progesterone type contraceptive; in the follicular phase (PROGEST-FOL), in the luteal phase (PROGEST-LUT) or amenorrhoeic (PROGEST-A), see Table 4-5 for the number of participants in each group.

4.2.3.1 Statistics

For both the absolute MFO (g/min) and the MFO relative to FFM (mg/kg FFM/min) the groups were normally distributed as assessed by distribution and QQ plots, and non-significant Shapiro-Wilk tests, with the exception of the groups E+P+A (n=1), PROGEST-FOL (n=2) and PROGEST-LUT (n=2) where these tests were not appropriate. Levene's test for equality of variance was met for both the absolute MFO and when expressed relative to FFM, therefore, differences between groups were explored using a one-way ANOVA, with Hochbergs GT2 post hoc tests to identify where the groups differences occurred. Values are means $\pm$ SD and statistical significance was set as $p \leq 0.05$.

4.2.3.2 Results

The One-way ANOVA revealed significant between group differences in both the absolute MFO and when expressed relative to FFM (p<0.001) with group differences highlighted in Table 4-7. Both the absolute MFO (g/min) and MFO relative to FFM (mg/kg FFM/min) were significantly (p<0.01) lower in the REG-FOL (0.40 $\pm$ 0.12 / 9.6 $\pm$ 2.6) than both the E+P+LUT (0.54 $\pm$ 0.14 / 12.5 $\pm$ 3.2) and the PROGEST-A (0.58 $\pm$ 0.19 / 13.2 $\pm$ 4.1), with a trend (p=0.06) for the absolute MFO to also be greater in PROGEST-A than REG-LUT (0.46 $\pm$ 0.13).

4.2.4 Discussion - Potential association of the menstrual cycle and hormonal contraceptive use on the maximal rate of fat oxidation during exercise on MFO

In agreement with prior research regarding the relationship between the menstrual cycle and substrate oxidation during exercise, a trend for a greater MFO during the luteal than the follicular phase of the cycle was observed (Nicklas, Hackney, and Sharp 1989; Jurkowski et al. 1978; Campbell, Angus, and Febbraio 2001; Zderic, Coggan, and Ruby 2001). Additionally, a greater MFO in women using hormonal contraceptives were seen than the MFO in regularly menstruating non-contraceptive users (McNeill and Mozingo 1981; Bonen, Haynes, and Graham 1991; Bemben et al. 1992), with an interaction, such that either amenorrhoeic women using a progesterone only form of contraceptive, or combined E+P users in the luteal

phase had a greater MFO than regularly menstruating women in the follicular phase. These findings will be discussed further below.

Our inconclusive finding of only a trend (p=0.06 and p=0.08) for a lower absolute and relative MFO respectively in the FOL vs the LUT phase unfortunately only adds to the inconsistency in the literature. When a difference has been reported in substrate oxidation by the phase of the menstrual cycle, a greater reliance of lipid and possibly protein has been seen in the luteal phase - when both oestrogen and progesterone levels are elevated. When oestrogen is administered in isolation to ovariectomized rodents, to men, amenorrhoeic women or women with pharmacologically suppressed ovarian hormone levels (D'Eon et al. 2002), then a greater reliance on fat oxidation has been documented. Although oestrogen supplementation doesn't seem to impact whole body lipolysis (Ruby et al. 1997), it seems to promote lipid oxidation through several other mechanisms such as through the upregulation of genes and transcription factors responsible for greater IMTAG storage (Fu et al. 2009), lipid membrane transport and mitochondrial biogenesis (Schulz et al. 2005; D'Eon et al. 2005) which should enhance oxidative lipid metabolism. Oestrogen supplementation also has an impact on carbohydrate metabolism, reducing hepatic glucose output, muscle glucose uptake and/or plasma metabolic clearance rate (Ruby et al. 1997; Carter et al. 2001; Devries et al. 2005). However, whilst oestrogen concentrations are greatest throughout the luteal phase of the menstrual cycle and so could explain the greater preference for lipid oxidation during this phase, progesterone levels are also elevated in comparison to the follicular phase, the independent impact of which is less clear, but could act antagonistically to oestrogen (D'Eon et al. 2002; Campbell and Febbraio 2001).

It was perhaps most surprising that we saw the greatest rate of lipid oxidation in the amenorrhoeic group who presumably (not measured) had a lower oestrogen level than both the FOL and LUT group. This group of women largely consisted of PROGEST only users (15/16), who had a similar MFO to those using a combined form (E+P) of contraceptive, both of whom had a greater MFO than those not using any contraceptive (REG). Taking this all into account, it implies that when the phase of the menstrual cycle is not accounted for, exogenous hormonal administration in general, dis-regarding the type *per se*, has a stronger positive association with MFO than no contraceptive use at all.

Considering the assumed oestrogen suppressive effect of the PROGEST contraceptive, it was hypothesized that this group would show the lowest MFO during exercise, it was therefore quite surprising to find the opposite. However, in support of this somewhat contentious finding, the groups did not differ in other known determinants of lipid oxidation, such as SRPAL, FFM, FM, $\dot{V}O_2$max, and dietary intake (data not shown). The only significant ($p<0.05$) difference between the groups was in carbohydrate intake (g/day), specifically, the REG-FOL (239.8 $\pm$ 70.1) vs E+P-FOL (310.5 $\pm$ 70.6) and the REG-FOL vs E+P+LUT (310.7 $\pm$ 67.6). The higher carbohydrate intake in the E+P-FOL and the E+P+LUT group would however be expected to be associated with a lower MFO, and so it is unlikely carbohydrate intake is influencing the findings. To my knowledge there are no human studies that have directly investigated the effects of progesterone administration in isolation on substrate oxidation during rest or exercise. This perhaps reflects the historically lower prevalence of PROGEST

type contraception's, with E+P more commonly prescribed. From studies in rodents however, showing oestrogen to enhance and progesterone diminish the maximal activity of key lipid oxidising enzymes and transcription factors that upregulate lipid oxidation (Campbell and Febbraio 2001; Campbell et al. 2003), our positive association of PROGEST and MFO that did not differ to the relationship of E+P with MFO, is somewhat surprising.

The human data to support or refute our positive association between lipid oxidation and PROGEST contraceptive use is limited. D'Eon *et al* (D'Eon et al. 2002) pharmacologically induced three tightly controlled hormonal environments representing; baseline (low oestrogen and low progesterone), oestrogen only (high oestrogen, low progesterone), and high oestrogen high progesterone to mimic the early follicular, late follicular and mid luteal phases, respectively. During exercise at 60% $\dot{V}O_2$max, greater plasma NEFA concentrations and greater rates of fat oxidation were reported in the high oestrogen condition (0.30 $\pm$ 0.04g/min) than either the low E+P (0.2 $\pm$ 0.04g/min) or the high E+P condition (0.2 $\pm$ 0.3g/min). Whilst this is in agreement with the suggestion that oestrogen promotes lipid oxidation, it is somewhat contradictory to trials reporting greater rates of lipid oxidation in the luteal phase of the menstrual cycle, when both oestrogen and progesterone would be elevated. Using 6,6-$_2$H labelled glucose, (D'Eon et al. 2002) were also able to attribute the reciprocal reduction in carbohydrate oxidation in the high oestrogen condition to a ~ 32% lower estimated muscle glycogen use (compared to the E+P condition) with a trend for lower plasma glucose use in the high oestrogen condition which would be in agreement with others (Ruby et al. 1997; Carter et al. 2001). However, these effects were not corroborated

in 2 similar studies, with the main distinction being that the 3 divergent hormonal environments were achieved naturally using regularly menstruating women (Horton et al. 2002; Horton et al. 2006). This investigational group saw no effect of oestrogen/progesterone during the different menstrual cycle phases on rates of substrate oxidation, plasma glucose, insulin, glycerol, NEFA, cortisol or catecholamines over a 90min exercise bout at 50% $\dot{V}O_2$max. Thus the independent impact of progesterone alone on substrate metabolism, gene expression and protein content of regulatory sites of lipid metabolism in humans is mixed and do not help clarify our findings.

The results and conclusions drawn from the analysis presented in this chapter must be viewed with caution. The simple approach used to split the menstrual cycle into 2 phases ignores the fluctuations in oestrogen and progesterone within the follicular and luteal phase (Figure 4-4), with oestrogen concentration 5 fold higher in the late follicular (days 10-14) than early follicular (days 0-7). Furthermore, with the substantial inter-individual and intra-cycle variation in hormonal concentration, without measuring hormonal concentration, simply using the calendar approach to determine the phase limits the validity of our results. Indeed, the intra-cycle hormonal variation, even when under “pharmacological control” is substantial and can lead to subjects being outside of expected ranges when quantified (D'Eon et al. 2002; Casazza et al. 2004). Indeed the inconsistency in the literature regarding the impact of either the phase of the menstrual cycle or hormonal contraceptive use on substrate metabolism is quite likely a reflection the large variation in hormone concentrations in typically small underpowered sample sizes (Syrop and Hammond 1987; Bao et al. 2003).

Furthermore, as highlighted in a recent review (Stachenfeld and Taylor 2014) many otherwise well controlled studies of substrate metabolism during exercise that compared the effects of a tri-phasic hormonal contraceptive, have incorrectly used the “dummy pill” days to compare users to the early follicular phase of regularly menstruating women. This model incorrectly assumes both endogenous and exogenous sex hormone concentrations are low and comparable (Casazza et al. 2004; Jacobs et al. 2005). However, although the administration of exogenous hormones is low over the 7 day “placebo” period, the endogenous oestrogens are highly variable over this time and so this might not be an appropriate model (Creinin et al. 2002).

Moreover, the approach taken to group contraceptive taking women into just 2 groups (either E+P or PROGEST) despite the differences in doses and types of synthetic oestrogen / progestogen was one of a pragmatism, based on the absence of detailed blood hormonal profiles. Notwithstanding these concerns, the finding of greater rates of lipid oxidation in amenorrhoeic hormonal contraceptive users is interesting and the absence of any human data of the independent effects of progesterone is worthy of follow up in future investigations.

4.3 Sex differences in the associations of dietary intake on the maximal rate of fat oxidation during exercise

From Table 4-1 in section 4.1 it was clear that there were many differences between the cohort of men and women tested in Chapter 4.1. Many of these differences were expected, for example, height and weight, but the range of differences extended to include physiological and nutritional variables known to impact substrate oxidation. With this in mind, and with the potential for sex differences in the metabolic response to dietary intake (see section 2.3.5), an additional analysis of the data from Chapter 4.1 was also conducted in each sex separately. With the appreciably smaller sample sizes in this analysis however, the findings must be interpreted with caution and regarded as exploratory. Nonetheless the main aim of this analysis was to provide potential insights and generate hypotheses for future follow up work.

4.3.1 Methods

For details regarding data collection and statistical analysis please see section 4.1.2. The only difference being that this analysis was conducted in men and women separately. A statistical "rule-of-thumb" when using multiple linear regression is to have >30 observations per predictor variable (Field 2013), therefore an extra ~ 60 participants would have been needed in each group thus the results below should be viewed in light of this.

For clarity, once the hierarchical linear regression equation has been calculated, each independent variable entered is given a standardised Beta coeffiecient value. The greater

the numerical value of this Beta coeffiecient (range -1.0:+1.0) implies a greater contribution to the explained variability. The direction of the association (i.e. positive or negative) with the dependent variable (e.g. MFO) is indicated by the positive or negative Beta coefficients.

4.3.2 Results

The results of the hierarchical regressions for the absolute MFO (g/min) (Table 4-6) and MFO relative to FFM (Table 4-7) can be seen below. For ease of comparison, both tables also include the original analysis with the entire cohort using sex as an independent predictor variable. The adjusted R^2 value in Table 4-6 & Table 4-7 represents the amount of variability in MFO explained at each of the hierarchical steps of the linear regressions. There was a greater amount of variability explained in women than men in both the absolute MFO (42.4 vs 32.9%) and MFO relative to FFM (23.3 vs. 15.3%).

For the absolute MFO (g/min) the hierarchical regressions for each sex both contained positive effects of $\dot{V}O_2$max and SRPAL. For men, the only additional significant independent contributor was carbohydrate intake (negative determinant), with the step 2 analysis explaining a modest additional 2.1% variance (trend for a change in R^2 [P = 0.06]). For women, both carbohydrate (negative determinant) and fat intake (positive) made additional contributions to the variance explained, with the step 2 analysis explaining a further 4.5% of the variance in MFO (g/min) (P<0.01).

With the MFO expressed relative to FFM the hierarchical regressions for each sex contained effects of SRPAL with significant effects of $\dot{V}O_2$max in men and FM in women. For men, the step 2 analysis showed a trend (P = 0.06) for a modest 2.7% increase in the amount of variability explained with protein intake the only significant independent contributor and a trend for carbohydrate intake (P = 0.06). For women the step 2 analysis explained a further 4.0% of the variance in MFO, with carbohydrate intake a significant negative independent predictor and a trend for fat intake (P = 0.08), which is directionally consistent with the significant positive association of fat intake in the analysis for absolute MFO (g/min).

Table 4-8. Hierarchical regressions for absolute Maximal Fat Oxidation (g/min) for the entire group (n 305) and separated by sex

		All Subjects (*n* = 305)		Men (*n* =150)		Women (*n* =155)	
Step	Independent Variables	Standardized Coefficients	Adjusted R^2	Standardized Coefficients	Adjusted R^2	Standardized Coefficients	Adjusted R^2
		Beta		Beta		Beta	
1			.435***		.308***[1]		.379***[1]
	$\dot{V}O_2$max (L/min)	.477***		.525***		.532***	
	SRPAL	.196***		.184*		.275***	
	Sex (M = 1, W = 2)	-.385***		-		-	
	FM (kg)	-.024		.015		-.053	
	FFM (kg)	-.091		-.082		-.162	
2			.462**		.329†[1]		.424**[1]
	$\dot{V}O_2$max (L/min)	.527***		.576***		.563***	
	SRPAL	.238***		.264**		.254**	
	Sex (M = 1, W = 2)	-.385***		-		-	
	FM (kg)	-.025		.0003		-.044	
	FFM (kg)	-.115†		-.098		-.171	
	Protein intake (g)	-.046		-0.91		.076	
	Carbohydrate Intake (g)	-.178***		-.208*		-.173*	
	Fat Intake (g)	.144**		.112		.180*	

*** *P* <0.001, ** *P* < 0.01, * *P* <0.05. † *P* <0.10. FFM, Fat Free Mass; SRPAL, Self-Reported Physical Activity Level; $\dot{V}O_2$max, maximal oxygen uptake.

[1] represents *P* value for change in R^2.

Table 4-9. Hierarchical regressions for absolute Maximal Fat Oxidation (mg/kg FFM/min) for the entire group (n 305) and separated by sex

		All Subjects (*n* = 305)		Men (*n* =150)		Women (*n* =155)	
Step	Independent Variables	Standardized Coefficients	Adjusted R^2	Standardized Coefficients	Adjusted R^2	Standardized Coefficients	Adjusted R^2
		Beta		Beta		Beta	
1			.174***		.126^{***1}		.193^{***1}
	$\dot{V}O_2$max (L/min)	.143*		.184*		.095	
	SRPAL	.261***		.224*		.322***	
	Sex (M = 1, W = 2)	.136**		-		-	
	FM (kg)	-.143**		-.092		-.183*	
2			.205**		.153$^{\dagger 1}$		.233^{*1}
	$\dot{V}O_2$max (L/min)	.196**		.246**		.140	
	SRPAL	.319***		.307***		.311***	
	Sex (M = 1, W = 2)	.136**		-		-	
	FM (kg)	-.158**		-.123		-.181*	
	Protein intake (g)	-.127*		-.206*		.070	
	Carbohydrate Intake (g)	-.189**		-.173†		-.214**	
	Fat Intake (g)	.147*		.097		.154†	

*** P <0.001, ** P < 0.01, * P <0.05. $^{\dagger}P$< 0.10. FM, Fat Mass; SRPAL, Self-Reported Physical Activity Level; $\dot{V}O_2$max, maximal oxygen uptake. ^{1} represents P value for change in R^2.

4.3.3 Discussion

This exploratory sex-specific analysis of the determinants of MFO during exercise showed that a greater proportion of the variability in MFO could be explain in women (42.4 & 23.2%) compared to men (32.9 & 15.3%) for both the absolute MFO and relative to FFM, respectively. The analysis reveals aerobic capacity and physical activity to be the dominant predictors of MFO and to a similar extent in either sex. The analysis also suggests the nutritional determinants of MFO differ between men and women, specifically; dietary carbohydrate intake is an independent predictor of MFO in both men and women, whilst dietary fat intake is an independent predictor of MFO in women only.

Aerobic fitness and SRPAL were the most prominent positive determinants of MFO having a similar magnitude of association in both sexes. The observation of enhanced fat oxidation with greater aerobic capacity has been reported many times (Bergman and Brooks 1999; Friedlander, Casazza, Horning, Huie, et al. 1998; Achten and Jeukendrup 2003). Indeed recent studies have demonstrated that increasing aerobic capacity through endurance training positively impacts MFO (Nordby et al. 2015; Rosenkilde et al. 2015). The positive association of aerobic capacity on MFO is likely a reflection of the exercise induced physiological and metabolic adaptations permitting greater lipid oxidation such as increased skeletal muscle capillarisation, mitochondrial mass and oxidative enzyme content, and greater fatty acid transport capacity across muscle and mitochondrial membranes (Holloszy and Coyle 1984; Coggan et al. 1992; Rosenkilde et al. 2015; Schenk and Horowitz 2006; Ingjer 1979). In previously sedentary individuals even low intensity exercise training that

does not induce improvements in $\dot{V}O_2$ max, and is more a surrogate for an increase in physical activity, can still lead to improvements in lipid oxidation (Schrauwen et al. 2002; Venables and Jeukendrup 2008), demonstrating the importance of physical activity *per se* regarding the factors underpinning metabolic health.

The negative relationship seen in both sexes between carbohydrate intake and MFO implies that an increase in habitual dietary carbohydrate intake would be associated with a reduction in lipid oxidation during exercise. Indeed, this is in agreement with controlled dietary interventions where marked increases in carbohydrate intake decrease fat oxidation during exercise (Bergstrom et al. 1967; Helge, Richter, and Kiens 1996; Støa et al. 2015; Phinney et al. 1983). This could simply reflect the direct influence of carbohydrate intake on its subsequent storage and availability for oxidation during exercise with similar directional responses seen in both men and women (Tarnopolsky et al. 2001; James et al. 2001).

The hierarchical regression equations from the sex specific models can be used to predict the degree and direction of change in MFO for a given change in <u>one</u> of the significant predictor variables, whilst keeping <u>all</u> other variables constant. For instance, in the women, an increase of 150 g/day of dietary fat whilst maintaining aerobic capacity, physical activity level, body composition, carbohydrate and protein intake, would be predicted to be associated with an increase in MFO by 0.15 g/min. The analysis reported here suggests a potential sex-difference in the relationship between habitual dietary fat intake and substrate oxidation during exercise, with a positive association seen in women only. For the men, the

dominant and negative association of carbohydrate and absence of a clear relationship with fat intake, is consistent with intervention studies in men of acute (1.5 days) hyper-energetic increases in dietary fat (fat supplementation) with a maintained carbohydrate intake having no effect on whole-body substrate oxidation rates during exercise at 55% $\dot{V}O_2$max (Decombaz et al. 2013; Zehnder et al. 2006). This short period of excess dietary fat was sufficient to elevate IMTAG pre-exercise by ~70% compared to a high carbohydrate low fat diet, with ~ three times greater rates of IMTAG utilisation. The greater IMTAG use was speculated to occur within the first hour of exercise, whilst plasma NEFA flux was likely low. Fat-adaptation protocols have been shown to require 5 days to achieve cellular adaptations favouring greater plasma NEFA extraction and use (Cameron-Smith et al. 2003; Yeo, Lessard, et al. 2008). It is possible therefore that either men do not respond to a period of fat supplementation or that a longer period of supplementation is required to achieve a similar shift in substrate oxidation augmenting both plasma NEFA and IMTAG utilisation, as is typically observed with high fat, low carbohydrate diets.

Alternatively, in contrast to the current and previous observations in men (Decombaz et al. 2013; Zehnder et al. 2006), the current analysis in women suggests that an independent increase in fat whilst keeping carbohydrate intake constant would be associated with an increase in fat oxidation during exercise, although to our knowledge this has not been investigated. Women have however been shown to be less susceptible than men to acute lipid-induced insulin resistance (Hoeg et al. 2011) providing some evidence of greater flexibility in metabolic response to periods of lipid excess. Thus with our sex specific hierarchical regression based on dietary intake data collected over 4 days, and a longer

duration than 1-2 days needed for true adaptations to high fat intake to occur, it is possible that 5 days of “fat supplementation” could augment fat oxidation in women. These observations and speculations of a sex-difference in the independent relationship of fat intake with MFO supports recent calls for further investigation of sex-specific influences of diet on exercise metabolism (Devries 2015).

This exploratory analysis also revealed that protein intake was associated with reduced MFO, relative to FFM, in men but not in women. This finding is difficult to explain, but could be related to the greater amount of protein consumed by the men, with this potentially impacting estimates of substrate oxidation that did not account for protein metabolism. If this were the case, however, one would expect a similar independent relationship between protein intake and absolute MFO, which was not seen. Thus, the reason for this inconsistent association of protein intake in men is unclear and may warrant further investigation.

To summarise, this exploratory sex specific analysis of the determinants of MFO during exercise, explained a greater proportion of the inter-individual variation in women compared to men. The most prominent predictors of MFO in both sexes were aerobic capacity and SRPAL with this likely reflective of the numerous metabolic adaptations to exercise training within muscle allowing a greater reliance on lipid. Carbohydrate intake was negatively associated with MFO consistently in both sexes whereas the independent and positive relationship of fat intake was more pronounced in women than men.

5 METABOLIC EFFECTS DURING EXERCISE AND ENDURANCE PERFORMANCE AFTER 5-DAYS EXPOSURE TO HIGH AND ELEVATED DIETARY FAT INTAKE IN ENDURANCE TRAINED WOMEN

5.1 Introduction

Since the pioneering work of Bergstrom and colleagues the close relationship between carbohydrate intake, muscle glycogen availability and fatigue during prolonged submaximal (60-85% $\dot{V}O_2$max) endurance exercise lasting >90mins has frequently been reported, with the development of fatigue occurring in close temporal association with a depletion in muscle glycogen (Bergstrom et al. 1967; Hargreaves, McConell, and Proietto 1995; Bangsbo et al. 1992; Gollnick et al. 1972). This association between muscle glycogen depletion and fatigue is often explained by an imbalance between skeletal muscle energy supply and demand with a compromised rate of ATP re-synthesis (Jensen and Richter 2011). More recently, it has even been proposed that glycogen is also a dynamic molecule that has a regulatory role in other distinct myocellular functions implicit to muscle contraction. For a comprehensive review regarding the role of glycogen in muscular fatigue see Ortenblad, Westerblad, and Nielsen (2013).

It is well established that altering the dietary intake of carbohydrate and fat results in differences in the circulating hormonal and metabolic milieu and the myocellular substrate storage profile, which can impact substrate utilisation during exercise and potentially exercise performance (Bergstrom et al. 1967; Burke et al. 2000). Therefore, substantial

research efforts have been made altering dietary macronutrient intake to maximise glycogen stores, whilst minimising its utilisation through promoting alternative (fat) energy sources (Burke 2015). Acute (<3days) high fat (60-65% EI), low carbohydrate (<20% EI) diets result in a reduction in muscle glycogen levels and an increase in whole body lipid oxidation (Burke 2015). A greater reliance upon the substrates that are elevated following the greater lipid intake, principally plasma NEFA and IMTAG is observed. While this is paralleled by a decline in muscle glycogen utilisation, this is most likely an artefact of the reduced muscle glycogen availability through the limited carbohydrate intake, with glycogen availability largely dictating its rate of degradation during exercise (Arkinstall et al. 2004; Jansson, Hjemdahl, and Kaijser 1982; Weltan et al. 1998). This often comes at the cost of lower glycogen availability and so exercise performance tends to suffer (Burke and Hawley 2002; Christensen and Hansen 1939; Bergstrom et al. 1967; Starling et al. 1997; Pitsiladis and Maughan 1999).

However, when consumed over a slightly longer period (>5days) there is evidence to suggest that metabolic adaptations to increased dietary fat intake occurs beyond the expected effect observed of fuel availability (Stellingwerff et al. 2006; Cameron-Smith et al. 2003; Stepto et al. 2002). For example, increased dietary fat intake for 5 days can almost double rates of fat oxidation over a 20min cycling bout at 70% $\dot{V}O_2$max (1.04 ± 0.07 vs 0.57 ± 0.07g/min), and although attenuated, lipid oxidation remains elevated following a day of carbohydrate restoration to normalise muscle glycogen stores (0.7 ± 0.05 g/min) or through other actions known to down-regulate fat oxidation such as the consumption of carbohydrate pre exercise or during exercise (Burke et al. 2000; Staudacher et al. 2001; Burke et al. 2002). The

elevation in lipid oxidation occurs with similar rates of plasma glucose oxidation (endogenous or exogenous if provided) suggesting a true sparing of muscle glycogen (Yeo, Lessard, et al. 2008; Burke et al. 2000; Burke et al. 2002).

The increased capacity to oxidise lipids following HF adaptation, however, comes at the expense of an attenuation in the capacity to oxidise carbohydrates, with a reduction in the active form of PDHa by >30%, at rest, submaximal and all out sprinting (Stellingwerff et al. 2006). This reduced PDH flux and pyruvate oxidation, suggests a reduced capacity or rate of glycogenolysis of the spared glycogen when needed the most (Fleming et al. 2004; Havemann et al. 2005). Thus the aforementioned dietary strategy rarely translates to an improvement in exercise performance (Havemann et al. 2005; Rowlands and Hopkins 2002b; Burke et al. 2000; Burke et al. 2002; Carey et al. 2001).

To summarise, a relatively acute (<3 day) period of exposure to a HF intake elevates lipid availability (plasma NEFA+IMTAG) (Zehnder et al. 2006; Zderic et al. 2004; Cameron-Smith et al. 2003; Tarnopolsky et al. 1995), but >5 days of exposure is needed to capitalise fully on the increased availability, which persist for ~ 36hrs even with glycogen restoration. However, this comes at the cost of an attenuation in the ability to use glycogen which is likely to be detrimental to exercise performance. Additionally, a caveat to the aforementioned HFLC studies is the manipulation of both dietary fat and carbohydrate within the same model. Thus, the metabolic adaptations and shifts in substrate oxidation to a HFLC intervention could be attributable to the addition of fat, the removal of carbohydrate or even a

combination of the two. An alternative approach would be to isolate the dietary change to just one macronutrient, keeping the intake of the other macronutrients constant. This hypercaloric model, only manipulates one macronutrient, making it clearer to determine the factor behind any shifts in substrate oxidation.

In this regard, two separate studies explored the impact of increasing dietary fat intake for 1.5days on the background of a high carbohydrate diet. The high carbohydrate (control) diet in these studies provided 7g carbohydrate/ kg BM/day with a small amount of fat (0.5g fat/kg BM/day), whereas the "fat supplemented" (hyper-caloric to EE) trial provided an equal amount of carbohydrate with an additional 2.5g fat/kg BM/day. Using magnetic resonance spectroscopy the authors were able to demonstrate that the fat supplemented trial elevated IMTAG over the control diet without compromising the ability to store or then use glycogen over a 3hr cycling bout at 50% of the maximum voluntary workload (Zehnder et al. 2006). However, this short period of high dietary fat intake did not increase the reliance on plasma sources of lipid to parallel the increase in IMTAG availability and use, resulting in no overall effect on whole-body lipid oxidation. This implies that carbohydrate restriction is necessary to see the typical augmentation of lipid oxidation to high dietary fat intake, although the short duration of exposure to high fat used may not have been sufficient to achieve adaptations reported to take 5 days. Therefore, it is proposed herein, that extending the period of exposure to high dietary fat (fat supplementation) alongside ample carbohydrate intake could increase the capacity to capitalise on the greater lipid availability, without compromising glycogen storage or the capacity for its degradation when required.

This could potentially manifest in overall greater rates of whole-body lipid oxidation and improvements in exercise performance.

As described in more detail in section 2.3.5, women compared to men, preferentially generate a greater proportion of energy through the catabolism of lipids over a wide range of exercise intensities and display a greater maximal rate of fat oxidation (Venables, Achten, and Jeukendrup 2005; Cheneviere et al. 2011). The aforementioned studies relating to HFLC diets, or HFLC with carbohydrate restoration, are almost exclusively carried out in men, with few if any well controlled intervention studies in women athletes. It is therefore not known to what extent a high fat diet may alter substrate oxidation during exercise in women, and what implications this may have on exercise performance. In comparison to men, women have also been shown to exhibit greater metabolic flexibility to periods of lipid excess (Hoeg et al. 2011), and so where dietary "fat supplementation" models have failed to alter substrate oxidation in men, they seem more likely to manifest in altered fuel use in women.

Additionally, despite the widespread use of hormonal contraceptives, particularly in an athletic population, there is a scarcity of data on the metabolic effects during exercise of the more recent 4th generation formulations. As discussed in section 4.2 we saw a greater MFO in women using a progesterone only form of hormonal contraceptive than regularly menstruating non-contraceptive users, but with a lack of comparative data, these results could not be discussed to any great extent. With the growing popularity of this form of contraceptive (Cea Soriano et al. 2014), the potential for a augmented lipid oxidising

response and the scarcity of metabolic and performance data, a comparative sub-set of progesterone only contraceptive users were also included in the study design.

The consumption of a high fat diet carries with it the potential for adversely effecting markers of cardiovascular disease (CVD) risk and inducing a state of reduced insulin sensitivity (Brøns et al. 2009). Although the risk of developing cardiovascular disease is substantially lowered by regular exercise in part by its favourable effect on the plasma lipid profile, the diet is still thought to have an independent effect (Miller et al. 2011). A HCLF diet, even in individuals regularly exercising has been associated with unfavourable alterations in the plasma lipid profile, specifically, decreases in HDL-C and considerable increases in hepatic synthesis and secretion of TAG rich VLDL-C (Mittendorfer and Sidossis 2001; Brown and Cox 1998; Thompson et al. 1984). In contrast, a diet moderate (35% EI) to high (>60% EI) in fat has been shown to have positive effects on certain lipoproteins, such as raising HDL-C and lower TAG, but also negatively raising LDL-C, total cholesterol and negatively impacting insulin sensitivity. There are only a few published studies of endurance-trained women investigating the effect of alterations in dietary fat or carbohydrate on markers of CVD risk and so the opportunity to explore this in the current investigation was also taken.

The recent advances in high-throughput technologies (transcriptomics, proteomics, and metabolomics) have enabled large-scale analyses not previously possible, with the capability to now characterize global alterations to the metabolic state of an organism associated to

different exposures such as exercise or diet (Astarita and Langridge 2013). Metabolomics refers to the quantitative assessment and characterisation of all metabolites (small molecules) within a biological system (Dunn, Broadhurst, Atherton, et al. 2011). The field of metabolomics is relatively new and perhaps currently underused in the area of exercise and nutrition, but represents a useful tool to explore the downstream effects of the interaction between perturbations in homeostasis due to diet or exercise and the genome, with biochemical information flowing from genome to transcriptome to proteome to metabolome. Metabolomics can be used to study the production and utilization of 1000s of metabolites at one snap-shot in time and can generate exploratory data mapping the association of phenotype with biological status of an organism. Thus, in the present study an untargeted mass-spec metabolomics approach was taken alongside more classical biochemical markers of carbohydrate and lipid metabolism to reveal in a more holistic approach, the extent to which dietary interventions perturbed homeostasis and what interaction this had during exercise.

To summarise, although the metabolic and performance effects of a HF diet in men have been well characterised, data in women is generally lacking. Compared to men, women possess a greater capacity for fat oxidation during submaximal exercise, along with the hormonal environment and underlying physiology that is potentially more conducive to magnifying a response to high dietary fat intakes. Lipid supplementation on the background of a carbohydrate replete diet has previously shown no impact on whole body rates of lipid oxidation in men, however the time course for adaptation to supplementation was short and the subjects tested (men) were not primed to oxidise fat. In light of this and the dearth of

metabolic data during exercise in women using progesterone only forms of contraceptive we set about to address these issues with the over-arching objective to better understand the impact of increasing lipid availability on substrate oxidation during exercise, with more specific purposes:

1. To determine if it is the restriction of carbohydrates or the provision of extra dietary fat, that is the stimulus evoking adaptations to a high fat diet promoting augmented rates of fat oxidation during exercise;
2. To better characterize the metabolic response to exercise following manipulation of dietary fat intake in regularly menstruating women and those using a progesterone only form of hormonal contraceptive;
3. To evaluate the impact of increased dietary fat on selected cardiovascular risk factors in endurance trained women;
4. To investigate the effect of greater dietary fat provision on endurance performance in trained women runners.

5.2 Methods

5.2.1 Participants

Between April 2015 and February 2016, forty healthy premenopausal Caucasian women were assessed for their eligibility to participate in the study, with fifteen completing the protocol having successfully met all inclusion criteria; generally healthy, endurance running based training at least twice weekly for > 12 months, $\dot{V}O_2max$ >50ml/kg BM/min, weight stable for > 6 months, either regularly menstruating (>11 cycles over the last 12months, no hormonal contraceptive use >12months) or using a progesterone only form of hormonal contraceptive. Data collection was completed by May 2016, see Figure 5-1 for a flow chart of participant recruitment and involvement in the study. Participants were recruited by postal notices, emails, social media, and contacting local running and triathlon clubs. Participants were excluded from taking part if they were: <18 or > 45 years old, BMI <17.0 or > 24.9 kg/m^2, taking any medication or supplements with the potential to interfere with normal metabolism (e.g., beta-blockers, insulin, bronchodilators, anti-inflammatory agents, thyroxine), a current or recent (within 30 days) smoker, engaged in prolonged periods of food abstinence or specific dietary restraint, pregnant, breast feeding or amenorrhoeic combined with not using progesterone only hormonal contraception. Participants provided written informed consent in accordance with the Helsinki Declaration of 1975 as revised in 1983 to take part in the study that was approved by the University of Birmingham local research ethics committee (Ref: ERN_15-0012) and was registered on clinicaltrials.gov (NCT02568592).

All volunteers were deemed to be healthy as assessed by a general health questionnaire (see section 7.5) with the characteristics at baseline of the 15 participants that completed the study (means ± SD): age, 34 ± 8 (yrs); height, 1.68 ± 0.06 (m); body mass, 58.1 ± 6.6 (kg); body fat 16.2 ± 3.6 (%); $\dot{V}O_2$max 3.19 ± 0.31 (L/min); $\dot{V}O_2$max 55.1 ± 2.5 (ml/kg/min). Eight women were regularly menstruating without using hormonal contraceptive, and 7 women were using a progesterone only form of contraceptive; mirena coil (Levonorgestrel) *n* = 3, Nexplanon (Etongestrel) *n* = 3, and Cerezarate (Desogestrel) *n* = 1. The women using a progesterone only form of contraceptive were well matched to the regularly menstruating women with no significant differences by way of an independent samples t-test seen in age, weight, body composition, aerobic capacity or habitual dietary intake.

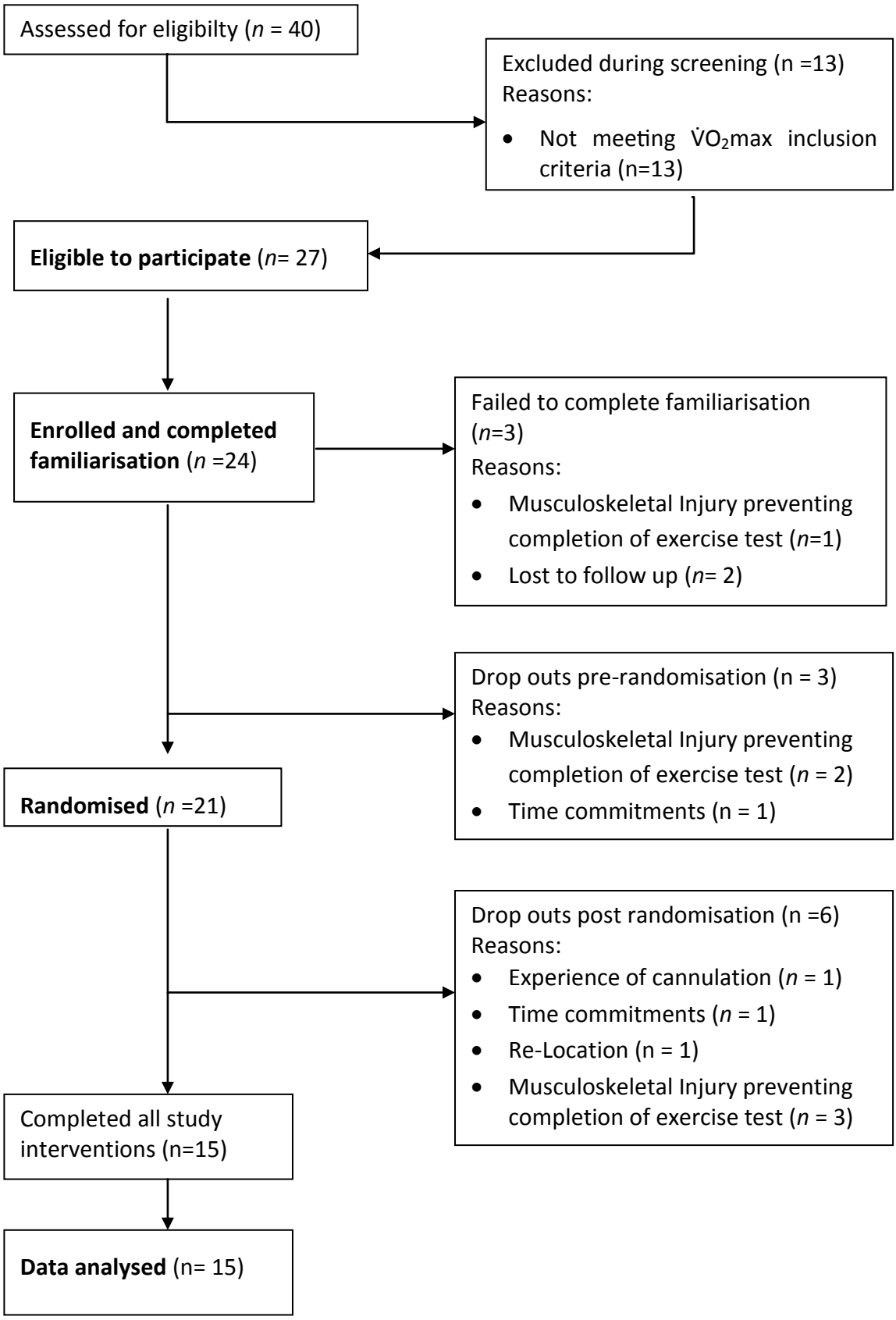

Figure 5-1 Participant flow chart

5.2.2 Study Design and general overview

This was a single-centre, randomised cross-over design study. The general overview can be seen in Figure 5-2. All testing took place at the University of Birmingham. Participants were provided with three different diets (see below) each for a period of five consecutive days with ~ one month wash-out period between interventions, the order of which was determined using on-line software to generate the randomization plan http://www.randomization in a randomised, partial-counterbalanced manner (i.e., the first 12 participants were counterbalanced, the remaining 4 (one of whom dropped out) were randomly allocated to 1 of the 6 possible trial order combinations with a three condition design).

In women regularly menstruating and not using hormonal contraceptives (*n* = 8), the dietary treatments were initiated so that the post-diet testing session commenced during the early to mid-follicular phase (day 1-10) of the menstrual cycle (average day 6 $\pm$ 2 for each dietary intervention). For logistical reasons it was not always possible for testing to follow from one menstrual cycle to the next as originally intended, with the average time between tests 1.8 ± 0.9 months. The women (*n* = 7) using progesterone only hormonal contraceptives, which typically suppress menstruation, started dietary interventions started approximately one month apart (1.3 ± 0.2).

5.2.2.1 Screening and Familirisation

Following informed consent and completion of a general health screening questionnaire, demographic data was recorded before undertaking an exercise test to determine the $\dot{V}O_2$

and running speed relationship along with a $\dot{V}O_2$max test (see below) to confirm eligibility. Within a few days of the screening visit, participants wore an Actiheart monitor (see below) (CamNtech, Cambridge, UK) for 5 consecutive days to monitor typical activity levels to determine energy requirements, and refrained from exercise on the 5th day. Habitual energy intake was assessed during this period using the multiple pass 24hr recall method (described in section 3.3.2). Immediately following the 5 days of baseline physical activity monitoring, participants attended the study site after a 10-12hr overnight-fast to be familiarised with the RMR measurement and the pre-loaded time trial (TT) test to be used in the main experimental trials (see below). Participants were familiarized with the testing procedures to ensure that the physiological and metabolic responses measured were as near normal and maximal as possible, not overly influenced by the novelty of the measurement and to minimise any possible learning effect regarding the TT (Doyle and Martinez 1998; Hopkins, Schabort, and Hawley 2001).

5.2.2.2 Main experimental trials

On day 1 of dietary intervention period, following a 10-12hr overnight fast except for water participants arrived at the laboratory between 6.00-09.00am for: anthropometric measurements (height, weight, waist circumference and skinfold measurements); before carrying out a treadmill based test to determine the running speed and $\dot{V}O_2$ relationship; followed by a $\dot{V}O_2$max; and then the completion of a 45-min treadmill run at 65% $\dot{V}O_2$max immediately before a 5km self-paced TT. Subjects were encouraged to drink at least 500ml of tap water upon waking before arrival at the laboratory for testing to minimise the

potential for differences in hydration status between dietary interventions. The running speed and $\dot{V}O_2$ relationship and maximal aerobic capacity were determined at the start of each dietary intervention period to monitor potential changes in fitness and to ensure that the steady state exercise was performed at the same relative exercise intensity allowing better between trial comparisons. The study diet commenced that day with the first meal provided on site and all remaining meals provided for the following 4 consecutive days (i.e., 5 days in total) during which the Actiheart activity monitor was worn throughout. Participants were instructed to consume only the meals provided and to follow similar physical activity / exercise patterns as captured during the familiarisation period, with exercise permitted during days 2-4 and only light activities on day 5. On day 6, following a 10-12hr overnight fast except for water, participants arrived at the laboratory between 6.00-09.00am for: anthropometric measurements (height, weight, waist circumference and skinfold measurements); RMR, a 90min treadmill run at 65% $\dot{V}O_2$max followed by a self-paced 5km TT. Blood samples were obtained at rest and during exercise.

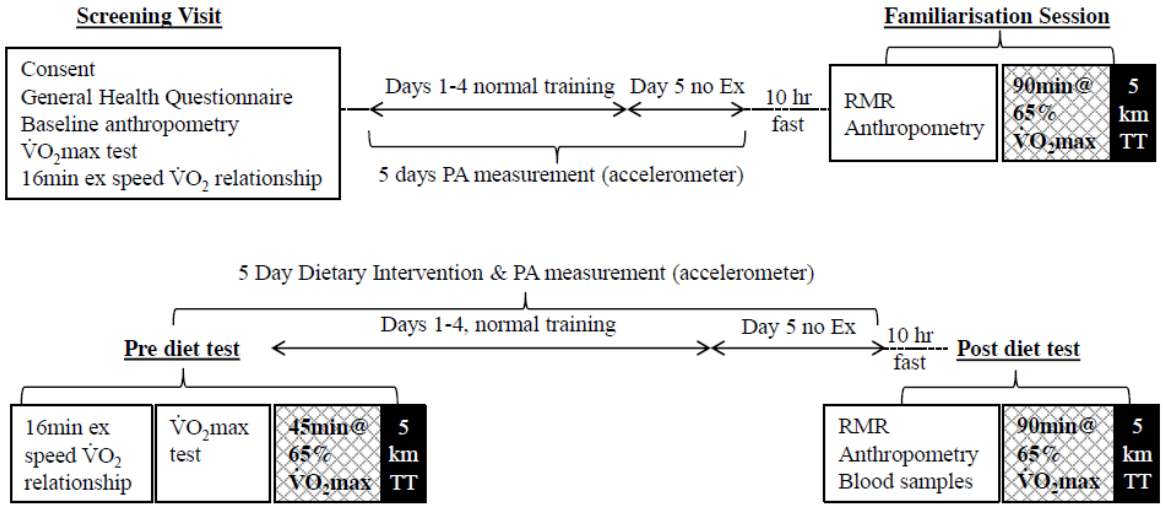

Figure 5-2. Schematic of study overview

5.2.3 Dietary intervention and analysis

The 3 dietary conditions and their respective macro-nutrient distributions as a percentage of total energy intake (carbohydrate/fat/protein) can be seen below in Figure 5-3, and were as follows; a “normal” diet (NORM) (50/35/15); a “high fat” (HF) (20/65/15), and a hypercaloric (130%) “normal diet + fat supplement” (N+HF) (50/65/15). The exact macronutrient composition of the study diets and the participant’s habitual dietary intake can be seen in (Table 5-2) and is described in more detail below. The NORM diet was designed to be fairly typical of an endurance type athlete and so acted as a control diet. The composition of the HF diet is consistent with recommendations regarding the definition of a “high fat” diet (Hawley 2011) and is sufficiently low in carbohydrates to enable adaptations to high fat diet to occur within 5 days as previously demonstrated in men (Cameron-Smith et al. 2003). The amount of extra fat in the N+HF diet was based both on previous overfeeding studies (Cornier et al. 2007; Van Proeyen et al. 2011) and was an amount considered achievable by the study population without giving cause for concern of weight gain.

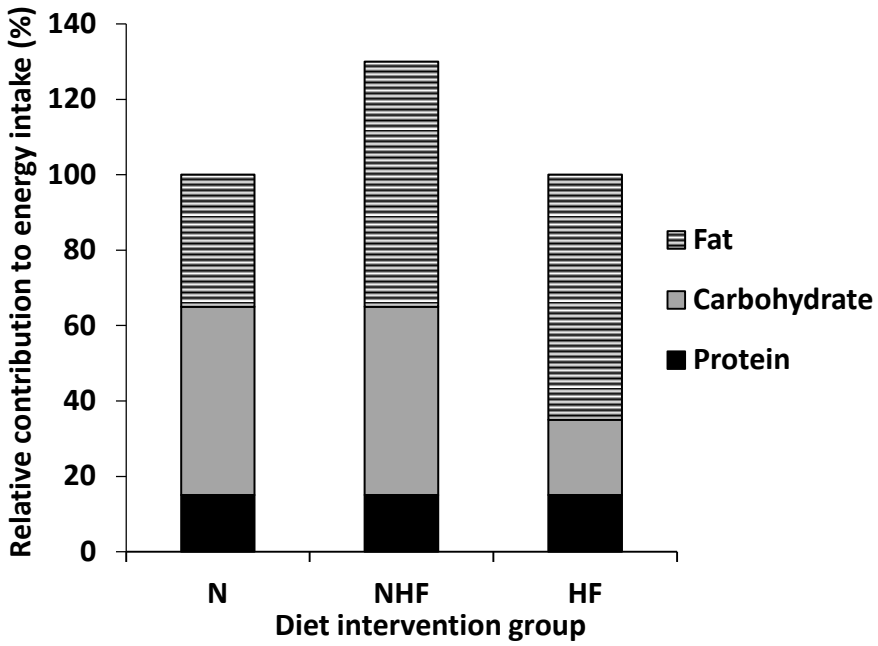

Figure 5-3 Relative contribution (%) of macronutrient intake to total energy intake of the study diets.

All study meals were prepared by GF from raw ingredients in the metabolic research kitchen at the University of Birmingham. Adjustments were made to recipes accounting for weight lost from pre to post through the cooking process (Chappell 1954). This allowed a greater level of control and precision over the composition of each meal or snack that the subjects consumed. Special considerations were made when composing the diets to make them as appetizing and healthy as possible in-light of the study population whom are notoriously restrictive eaters especially regarding the avoidance of high fat foods (Horvath et al. 2000). The subjects were instructed to consume all meals provided in their entirety, and were additionally asked to weigh and record any left-overs / unconsumed prescribed foods. In general, the diets were well tolerated with only minimal amendments made to the prescribed diets. Thus the overall impact to the consumed dietary intakes of the study diets

was negligible (± 1-2% of planned intake) regarding either total intake or the macronutrient distributions.

Habitual dietary intake was determined by the multiple pass 24hr recall method, which utilises prompting to reduce food omission (Hebert et al. 2002) and was performed on 3 occasions to include two weekdays and one weekend, with 3 days considered the minimum number of days to reliably estimate energy and macronutrient intake (Hebert et al. 1998). The Foods Standards Agency Food Portion Sizes booklet (Nelson et al. 1997) was also used to aid in the estimation of food portion sizes, with data analysed using the computer software Dietplan 6.70.67 (Forestfield Software Ltd. Horsham, West Sussex, UK).

5.2.4 Physical activity and energy expenditure monitoring

5.2.4.1 Daily physical activity

Throughout the 5 day dietary intervention period, subjects wore a combined accelerometer and heart rate monitor (CamNtech, Cambridge, UK) described in more detail in section 3.4.2) and elsewhere (Brage et al. 2005). Briefly, in accordance with the manufacturer's instructions, this monitoring device was attached to the chest by long-term ECG pads (Red Dot solid gel electrodes, 3M, Bracknell, UK) simultaneously measuring heart rate and uni-axial accelerometer counts which are combined using a branched equation model to determine physical activity energy expenditure (Brage et al. 2004). Recordings were made in 30 sec epochs throughout the wear time and the raw data was cleaned and processed using Actiheart software (version 4.0.116; CamNtech Ltd, Cambridge UK). Individual heart rate-$\dot{V}O_2$

calibration curves were established for the individual relationship between heart rate and energy expenditure enabling the use of the "Group Act/Ind HR+stress" energy model from the Actiheart software for improved estimates of energy expenditure (Assah et al. 2011). Total daily energy expenditure was the sum of RMR (see below), Actiheart derived physical activity energy expenditure and an estimated diet induced thermogenesis (10% daily EI (Westerterp 2004).

5.2.4.2 Resting Metabolic Rate

Resting Metabolic Rate was measured in the overnight fasted state by using an indirect calorimeter fitted with a ventilated hood (GEMNutrition Ltd. Cheshire, UK). Ambient room temperature was set to a comfortable (21-24°C) in a dimly light room, subjects lay awake, undisturbed and motionless in a supine position, for 30mins whilst exhaled gases were collected over a constant airflow of 40L/min. $\dot{V}O_2$ and $\dot{V}CO_2$ measurements were averaged over 30sec periods, with values discarded from the first 10 and last 2mins, with the average of the remaining 18mins used to determine the RMR provided the CV for $\dot{V}O_2$ and $\dot{V}CO_2$ was <10%. RMR was calculated using the Weir equation without correction for urinary nitrogen (Weir 1949). The gas analysers were automatically calibrated immediately before each use by using certified reference gases (1.00% CO_2, 21.00% O_2, balance Nitrogen) (BOC Gases, Surrey, UK). Unfortunately, due to equipment failure, RMR measurements were only available on 8/15 participants and so RMR was estimated using the Harris-Benedict equation (Harris and Benedict 1918) for the remaining 7 participants. In those subjects where RMR could be measured, an independent samples t-test was used to compare the measured RMR

values (1586 ± 221 kcal) to the Harris-Benedict calculated values (1514 ± 219 kcal) and did not significantly differ (*P*=0.28).

5.2.5 Anthropometry and Body Composition Assessment

After voiding and whilst wearing minimal clothing, participants were weighed to the nearest 10g (OHaus, Champ II scales, USA) and height was measured to the nearest cm (Stadiometer, Seca, UK). Waist circumference was measured to the nearest mm with the tape measured midway between the uppermost border of the iliac crest and the lower border of the costal margin (rib cage). Body composition measurements were performed by an International Society for the Advancement of Kinanthropometry (ISAK) Level 1 Accredited Anthropometrist and followed International Standards for Anthropometric Assessment. Skinfold thickness was measured to the nearest 0.1 mm in duplicate (triplicate when the second measure was ≥5% different to the first at which point the median value was used) using calibrated callipers (Harpenden, West Sussex, England) at 4 skinfold sites; triceps, subscapular, biceps and supraspinale, the sum of which was used to estimate body composition (Davidson et al. 2011).

5.2.6 Exercise Tests

All exercise tests were carried out on a motorised treadmill (HP cosmos, Quasar, Nussdorf-Traunstein, Germany), with the initial starting gradient set to 1% in all tests to reflect the oxygen cost of outdoor running (Jones and Doust 1996). Heart rate was recorded continuously throughout each test by telemetry using a heart rate monitor (Polar M400,

Polar Electro Ltd, Oy, Finland). Environmental conditions during testing were controlled to minimise the possible effect of thermal stress and any impact this may have on substrate metabolism (O'Hearn et al. 2016), with: relative humidity 47±8%; temperature 20±2°C. An electronic fan was positioned behind participants for use upon request. Water was provided ad-libitum but monitored and did not significantly differ throughout all the exercise trials averaging 458 $\pm$ 275, 467 $\pm$ 319 and 364 $\pm$ 315ml in the post diet NORM, HF and N+HF trial respectively.

Either a mouthpiece and a nose clip or a face-mask with head-strap (7450 V2, Hans Rudolph, Missouri, USA) was securely fitted to the subject to allow breath-by-breath respiratory measurements (minute ventilation, $\dot{V}E$; oxygen consumption, $\dot{V}O_2$; carbon dioxide production, $\dot{V}CO_2$) to be recorded throughout the exercise tests (besides the TT where no such measurements were made) using an automated gas analysis system (Oxycon Pro, CareFusion UK Ltd, Basingstoke, UK). The gas analysers were calibrated immediately before each exercise test according to the manufacturers recommendations using calibration gases (5.07% CO_2, 14.79% O_2) (BOC Gases, Surrey, UK), and the volume transducer was manually calibrated with a 3 litre bi-directional syringe (Jaegar, Wuerzberg, Germany).

5.2.6.1 Pre-diet, $\dot{V}O_2$ running speed relationship

On the treadmill, participants ran at 4 incremental submaximal speeds, each for a period of 4 minutes with expired air continuously monitored. The average expired air over the final

minute of each stage was used to calculate the linear relationship between $\dot{V}O_2$ and running speed.

5.2.6.2 Pre-diet, $\dot{V}O_2$max test

Following the test to determine the relationship between $\dot{V}O_2$ and running speed, a 5min rest was given before initiating a graded treadmill exercise test adapted from that used by Scrimgeour *et al* (Scrimgeour et al. 1986) to determine maximal aerobic capacity. The treadmill was started at 8km/h and the speed increased every minute by 1km/h until either an RER of 1 was reached or the subject indicated difficulty maintaining the speed at which point the treadmill gradient was increased by 1% every minute until volitional exhaustion. Breath by breath measures were recorded throughout to measure $\dot{V}E$, $\dot{V}O_2$ and $\dot{V}CO_2$, with the highest rolling 60 second average $\dot{V}O_2$ measurement considered to be maximal ($\dot{V}O_2$max) if 2 of the 3 following conditions were met: 1) a levelling off of $\dot{V}O_2$ with further increasing workloads (an increase of ≤2 mL/kg/min); 2) a heart rate within 10 beats/min of the age-predicted maximum (206 beats per minute – 0.88(age)) (Gulati et al. 2010); or 3) a respiratory exchange ratio of >1.0.

5.2.6.3 Pre-diet, 45 Pre-loaded 5km Time Trial test

Following a 5min recovery from the $\dot{V}O_2$max test an adapted version of the preloaded treadmill based time trial test used by Russell *et al* (Russell et al. 2004) was started. This protocol has been shown to be a reliable measure of endurance performance, with the CV to complete repeated 90min preloaded 10km TT's in women runners being 1.26%. The shorter

45min preloaded 5km TT used here served 2 purposes; Firstly, to act as an additional familiarisation session with it demonstrated on numerous occasions that a familiarisation session is required to reduce the variation within subjects during laboratory based performance tests, with variability reduced further with additional familiarisation sessions (Andrews et al. 2003). And secondly to lower the subject's glycogen stores in an effort to standardise the subjects muscle substrate availability prior to the dietary treatment.

Heart rate and breath by breath indirect calorimetry were recorded throughout the 45min steady state treadmill run at 65% of the recently measured $\dot{V}O_2$max, with RPE measured every 15mins (Borg 1982). Upon completing the steady state run, a short toilet break was enforced and stretching permitted, before the 5 km self-paced treadmill TT was initiated, during which only heart rate was recorded. Participants were given the same instructions prior to each TT, and were asked to cover the 5 km in as short a time as possible. Participants had full control over the treadmill speed operated either by a hand-held remote or treadmill mounted display that only showed the distance covered. There were no visible clocks or other means of monitoring time / speed available to the subjects. No encouragement or distractions (audio/visual) were permitted. Blinding the subjects to the dietary treatment conditions was not possible. To reduce bias on the TT, subjects were not informed of their performances until the completion of the study (Doyle and Martinez 1998).

5.2.6.4 Post diet, 90 Pre-loaded 5km Time Trial test

Approximately 10mins after collection of the resting blood sample (see below) the 90min steady state run at 65% of the $\dot{V}O_2$max measured at the start of each dietary intervention was initiated. A blood sample was drawn at 30, 60 and 90mins of exercise along with measures of heart rate and RPE. Breath by breath measurements of $\dot{V}O_2$/ $\dot{V}CO_2$ were recorded over a 5min period every 30mins, with the average values taken during the final minute used to calculate rates of substrate oxidation using the stoichiometric equations of Frayn (Frayn 1983) under the assumption of negligible urinary nitrogen losses. Upon completion of the 90min run, the cannula was removed and a short toilet break was enforced and stretching permitted, before the 5 km self-paced TT was initiated under the same conditions as the pre diet TT described above.

5.2.7 Post diet, blood sampling and analysis

Venous blood samples were drawn through an indwelling cannula (20g IV catheter, BD Venflon, Plymouth, UK) inserted into an antecubital vein connected to a 150cm polyethylene extension line (V-Green I.V. Extension Line, Vygon, Swindon, UK). A 3-way stopcock (BD Connecta, Plymouth, UK) was attached to the extension line, allowing blood samples to be drawn whilst the participant was running on the treadmill without the need for stopping or substantial changes in running gait. A 20ml resting sample was drawn immediately following the RMR measurement whilst the subject remained in a supine position, with 10ml samples taken at 30,60 and 90mins of the steady state exercise. The cannula was kept patent throughout the duration of insertion by regular flushing of a 0.9% sodium chloride (B Braun,

Melsungen, Germany). Whole blood was drawn into EDTA or lithium heparin tubes that were immediately stored on ice with additional blood drawn was into serum tubes and left to clot at room temperature for ~60mins, after which all tubes were centrifuged at 1361g (3000rpm) for 15mins at 4°C. Aliquots containing plasma or serum were then flash frozen in liquid nitrogen and stored at -80°C until analysed.

Sample analysis was performed in duplicate using enzymatic colorimetric assays for plasma glucose (Glucose Oxidase, Instrumentation Laboratories, Cheshire, UK), NEFAs (NEFA, Randox, London, UK), glycerol (GLY, Randox, London, UK) lactate (L-Lac, Randox, London, UK), triglycerides, (Triglycerides, Instrumentation Laboratories, Cheshire, UK), Total cholesterol, (Cholesterol, Instrumentation Laboratories, Cheshire, UK), HDL cholesterol, (HDL cholesterol, Instrumentation Laboratories, Cheshire, UK) and LDL cholesterol (LDL cholesterol, Instrumentation Laboratories, Cheshire, UK) using an ILAB 650 clinical chemistry analyser (Instrumentation Laboratories, Cheshire, UK). Serum insulin concentration was determined in duplicate by radioimmunoassay using a commercially available kit (HI-14 K Human Insulin, Millipore, Hertfordshire, UK). Finally, serum oestrogen and progesterone concentrations were measured in separate analyses quantified by immunoassays using a Roche e602 unit on a Cobas 8000 modular analyser (Roche Diagnostics Ltd, Rotkreuz, Switzerland).

5.2.8 Metabolomics

The metabolomics sample preparation, analysis and data processing was carried out by the Phenome Centre Birmingham. A thorough description of the methods for this process is provided in the Appendix (section **7.6**) produced by Dr Giovanny Bianco and Dr Warwick Dunn. A shorter adapted version is provided below.

5.2.8.1 Sample Preparation and Metabolite extraction / Untargeted metabolomics

Following a monophasic extraction protocol, plasma samples were centrifuged at 14,000 x *g* for 15 minutes at 4°C and 150 µL of the supernatants were then transferred into separate glass HPLC vials for Ultra High Performance Liquid Chromatography-Mass Spectrometry (UHPLC-MS) analysis. UHPLC-MS analysis was performed applying an Ultimate3000 RSLC UHPLC system coupled to an electrospray Q-Exactive Focus mass spectrometer operating in both positive and negative ion modes. Mass calibration was performed for each polarity immediately before each analysis batch.

Two different columns were used for metabolite separation, for polar compounds, an Accucore-150-Amide-HILIC (100 x 2.1 x 2.6 µm, Thermo Scientific) was used with column temperature set to 30°C and the flow rate was 500 µL min^{-1}. Separation of lipids species was performed using a Hypersil Gold C_{18} column (100 x 2.1 x 1.9 µm, Thermo Scientific) with the column maintained at 55°C and the flow rate set at 250 µL min.

5.2.8.2 Data pre-processing and analysis

UHPLC-MS raw data files (.RAW) were converted to a .mzML format by using the MS-Convert software available in the ProteoWizard package (available at http://proteowizard.sourceforge.net/tools.shtml). Data deconvolution was then performed using XCMS to provide a 2D matrix of chromatographic peaks responses where each peak was defined by the m/z ratio and retention time (Dunn et al. 2008). This 2D matrix was exported as a .csv file for data analysis. Post metabolite annotation, data filtering was performed to remove all metabolite features with more than 40% missing values for all QC samples.

5.2.9 Statistical analyses

Data were analysed using the SPSS statistical package for Windows, version 23.0.0 (SPSS, Chicago, IL, USA). Data were checked for normality using distribution plots and the Shapiro-Wilk test and is presented as mean ± SD with statistical significance accepted at $P \leq 0.05$. Summary data including; AUC, total substrates oxidized, RER, RPE, energy expenditure and the TT data were assessed for differences using a repeated-measures one-way ANOVA. The effects of diet on responses during exercise were evaluated using a two-way (diet X time) ANOVA with repeated measures with significance accepted at $P \leq 0.05$ for all analyses. Violations to assumptions of sphericity were adjusted using the Greenhouse-Geisser correction factor. Significant interactions were followed up using post hoc tests with Bonferroni adjustments for multiple comparisons.

As identified in section 4.2, the literature is unclear whether hormonal contraceptive use has an impact on substrate oxidation during exercise. For this reason, all variables were compared by an independent samples t-test for differences between contraceptive users and non-users. The only significant difference ($p<0.05$) was seen in the oestrogen concentration in the HF trial (lower in contraceptive users). Therefore, with the general lack of effect concerning contraceptive use, the groups were collapsed across all conditions however for specific variables of interest contraceptive use was still used as a between groups factor.

Oestrogen and progesterone data were log-transformed accounting for non-normal distribution and substantial outliers. A repeated-measures one-way ANOVA showed no differences ($P> 0.05$) in the hormone concentrations (between diets with contraceptive use as a between subjects factor or when the groups were collapsed), therefore no further analysis with these variables as covariates were undertaken.

The area under the plasma / serum metabolite concentration versus time curve (AUC) was used a summary statistic for the blood metabolite responses over exercise and were calculated using the trapezoidal rule (Matthews 1988). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the formula of (Matthews et al. 1985) (fasting insulin (μU/ml) x fasting glucose (mmol/L)/22.5)

Post-hoc power calculations were computed using G*Power version 3.1.9.2 (Heinrich Heine, University Dusseldorf, Germany).

Statistical analysis for the metabolomics data was performed by the Phenome Centre Birmingham using the software package MetaboAnalyst (Xia et al. 2012). There were no missing value imputations made. The normalisation to the sum of the peak areas for each sample were used and no transformations or scaling were performed. Where two metabolite classes were compared, a non-parametric Mann-Whitney U test was performed with correction for false discovery rate (FDR) applying the Benjamini-Hochberg method. Where three metabolite classes were compared a parametric one-way ANOVA test was performed with correction for FDR applying the Benjamini-Hochberg method. Fold changes were calculated applying the mean response for each class.

5.3 Results

5.3.1 Anthoropometric and physiological characteristics

There were no significant differences in aerobic capacity, body mass or composition at the start of 3 different dietary interventions (see Table 5-1) or compared to baseline. Importantly, this indicates that the dietary interventions were started under similar fitness and physiological conditions, with any differences in the post diet measurements a reflection of the dietary intervention.

Body mass was maintained from the pre to post measurements on the NHF diet, whereas there was a slight decrease from pre-post in both the NORM and the HF diets with the loss significantly ($p<0.05$) greater in the HF than NORM and the NHF. The small loss of weight in

both the NORM and HF diets arose from a loss of both body fat and FFM resulting in a significant change in body fat (%) in the HF compared to the NHF.

Table 5-1 Participant characteristics

Parameter	NORM	HF	NHF
Pre diet mass (kg)	58.6 ± 6.5	58.8 ± 6.7	58.7 ± 6.3
Post diet mass (kg)	58.0 ± 6.4^{*b}	57.3 ± 6.4^{*ac}	58.6 ± 6.2^{b}
Body mass change (kg)	-0.63 ± 0.56	-1.45 ± 0.61	-0.14 ± 0.68
Pre BMI	20.7 ± 1.8	20.7 ± 1.8	20.7 ± 1.8
Post BMI	20.4 ± 1.7	20.2 ± 1.7	20.6 ± 1.7
Pre WC	68.9 ± 3.5	68.8 ± 3.0	69.2 ± 3.7
Pre body fat (%)	16.6 ± 3.6	16.5 ± 3.8	16.7 ± 4.0
Post body fat (%)	16.4 ± 3.6	15.7 ± 3.9^{*c}	16.8 ± 3.7
Body fat (%) change	-0.2 ± 0.6	-0.8 ± 0.7	0.1 ± 0.6
Pre FM (kg)	9.9 ± 3.0	9.9 ± 3.1	9.9 ± 3.1
Post FM (kg)	9.6 ± 2.9*	9.1 ± 3.0^{*ac}	9.9 ± 2.9
Pre FFM (kg)	48.8 ± 4.3	48.9 ± 4.4	48.8 ± 4.2
Post FFM (kg)	48.4 ± 4.2*	48.2 ± 4.1^{*c}	48.6 ± 4.3
FFM change	-0.4 ± 0.6	-0.7 ± 0.6	-0.2 ± 0.7
$\dot{V}O_2$ max (L)	3168 ± 345	3123 ± 353	3157 ± 383
$\dot{V}O_2$ max (ml/kg/min)	54.1 ± 2.8	53.2 ± 2.8	53.8 ± 3.4

Data are means ± SD for all parameters (*n* = 15). Superscript symbols/letters represent; * significantly different pre to post diet, ^{a} significantly different to NORM (P<0.05), ^{b} significantly different to HF (P<0.01), ^{c} significantly different to N+HF (P<0.01). BMI; body mass index, WC, waist circumference; FFM, fat free mass; FM, fat mass; $\dot{V}O_2$ max, maximal aerobic capacity. NORM, normal diet; HF, high fat diet; NHF, Normal + extra fat diet intervention.

5.3.2 Dietary intake and physical activity level

The NORM diet was very similar to the estimated habitual dietary intake, differing only by a lower amount of fibre consumed (g/day) and a lack of alcohol in the study interventions

(Table 5-2). The study diets achieved their objectives regarding a well matched intake (absolute amounts) of fibre and protein across all 3 diets, with the NORM and NHF diet differing only by the amount (g/day) of fat and thus energy (attributed entirely to the 30% greater fat intake). The HF diet had a significantly higher intake of total fat and lower intake of carbohydrate compared to both the NORM and the NHF diets. Although statistically different, the marginally greater fat intake in the HF diet compared to the NHF amounted to only ~ 5g/day difference, and is considered unlikely to be physiologically meaningful. Importantly there were also no differences in the estimated energy expenditure between the study diet conditions or when compared to the baseline period (Table 5-2), indicating that as requested, the subjects maintained a similar level of daily activity and exercise training over the course of the study.

Table 5-2. Calculated daily intake and expenditure of energy and nutrients

Variable	Dietary intervention			
	Habitual	NORM	HF	NHF
Protein (g/day)	99 ± 16	100 ± 14	99 ± 12	101 ± 13
Fat (g/day)	104 ± 33	99 ± 12	191 ± 21[abd]	186 ± 20[abc]
carbohydrate (g/day)	333 ± 94	359 ± 47	138 ± 15[abd]	358 ± 39
Alcohol (g/day)	9 ± 13	-	-	-
Fibre (g/day)	31 ± 7[bcd]	22 ± 7	20 ± 3	23 ± 8
Protein (%)	14.9 ± 2.6	15.0 ± 0.4	14.9 ± 0.3	11.6 ± 0.3[abc]
Fat (%)	34.3 ± 4.6	33.2 ± 0.8	64.3 ± 0.7[abd]	48.4 ± 0.8[abc]
carbohydrate (%)	46.0 ± 6.2	50.1 ± 0.3	19.3 ± 0.6[abd]	38.7 ± 0.4[abc]
Alcohol (%)	2.5 ± 3.2	-	-	-
Fibre (%)	2.3 ± 0.4[bcd]	1.7 ± 0.4[ad]	1.5 ± 0.3[a]	1.3 ± 0.4[ab]
Protein (g/kg BM)	1.7 ± 0.3	1.7 ± 0.2	1.7 ± 0.2	1.7 ± 0.2
Fat (g/kg BM)	1.8 ± 0.6	1.7 ± 0.1	3.3 ± 0.3[ab]	3.2 ± 0.3[ab]
carbohydrate (g/kg BM)	5.7 ± 1.6	6.1 ± 0.6	2.4 ± 0.3[abd]	6.1 ± 0.5
Alcohol (g/kg BM)	0.2 ± 0.2	-	-	-
Fibre (g/kg BM)	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
Energy Intake (kcal/day)	2708 ± 631	2683 ± 340	2673 ± 292	3463 ± 384[abc]
Energy Expenditure (kcal/day)	2825 ± 283	2698 ± 350	2658 ± 386	2771 ± 259
Energy Balance (kcal/day)	118 ± 755	-15 ± 288	14 ± 278	692 ± 352[abc]

Data provided are means ± SD (*n* = 15). Superscript letters signify; [a] significantly different to Habitual (P<0.01), [b] significantly different to NORM (P<0.01), [c] significantly different to HF (P<0.01), [d] significantly different to N+HF (P<0.01).

5.3.3 Submaximal steady state exercise

5.3.3.1 Exercise intensity, speed, heart rate, rating of percieved exhaustion

There were no significant differences between the diets in the relative or absolute intensity that the submaximal treadmill test was performed at with similar treadmill speeds between the interventions (see

Table **5-3**). There were no differences between the trials in the change in weight from the start to the finish of exercise (NORM -0.6 ± 0.4kg; HF -0.3 ± 0.9kg; NHF -0.6 ± 0.7kg) implying a similar level of environmental stress and sweat rates.

Table 5-3 Selected parameters of interest over the 90min submaximal exercise bout

Parameter		NORM	HF	NHF
Time				
0-30 (mins)	$\dot{V}O_2$ (L/min)	2076 ± 263	2111 ± 253	2113 ± 234
	$\dot{V}O_2$ (ml/kg/bm)	35.8 ± 2.6	36.9 ± 2.2	36.1 ± 2.2
	Heart rate (beats/min)*	131 ± 12	134 ± 11	133 ± 9
30-60 (mins)	$\dot{V}O_2$ (L/min)	2095 ± 260	2094 ± 252	2097 ± 227
	$\dot{V}O_2$ (ml/kg/bm)	36.2 ± 2.7	36.6 ± 2.3	35.9 ± 2.4
	Heart rate (beats/min)*	135 ± 11	136 ± 10	137 ± 8
60-90 (mins)	$\dot{V}O_2$ (L/min)	2088 ± 279	2080 ± 249	2105 ± 269
	$\dot{V}O_2$ (ml/kg/bm)	36.0 ± 2.5	36.3 ± 2.6	36.0 ± 2.6
	Heart rate (beats/min)*	136 ± 13	138 ± 10	138 ± 9
0-90 (mins)	$\dot{V}O_2$ (L/min)	2087 ± 266	2095 ± 250	2105 ± 249
	$\dot{V}O_2$ (ml/kg/bm)	36.0 ± 2.5	36.6 ± 2.3	36.0 ± 2.0
	% $\dot{V}O_2$ max	65.8 ± 2.8	67.1 ± 2.5	66.8 ± 2.4
	Heart rate (beats/min)	134 ± 12	136 ± 10	136 ± 8
	RER	0.83 ± 0.03	0.77 ± 0.03 [a]	0.82 ± 0.03
	Running speed km/h	9.9 ± 0.7	10.2 ± 0.8	10.3 ± 1.4
	RPE	11 ± 2	11 ± 1	10 ± 1

Data provided are means ± SD (*n* = 15). Superscript symbols signify; [a] significantly different to NORM and NHF (P<0.01), * trend (*p*=0.07) for main effect of diet. RER; respiratory exchange ratio, RPE; rating of perceived exertion.

Other than the RER which was significantly lower in the HF trial than the NORM and the NHF trial (p<0.01) there were no significant differences between any of the parameters shown in Table 5-3. Nonetheless a main effect of time (p<0.05), was apparent, with cardiovascular drift seen with a gradual increase in heart rate as time of the exercise bout increased, along with a trend (p=0.07) for a main effect of diet, with heart rate appearing to be lower in the NORM than the HF and NHF diets.

5.3.3.2 Substrate oxidation

The rates of fat oxidation were significantly greater, and carbohydrate significantly lower in the HF trial than the NORM and the NHF ($p<0.01$), with no differences seen between the NORM and NHF. The relative contribution of fat oxidation to energy expenditure was also therefore significantly ($p<0.01$) greater in the HF trial than both the NORM and NHF (76, 57 and 59% respectively). There was also a significant main effect of time across all trials with rates of fat oxidation marginally (0.04g/min) greater at 90 than 30mins and the reciprocal relationship regarding carbohydrate oxidation being greater (0.11g/min) at 30 than 90mins ($p<0.05$).

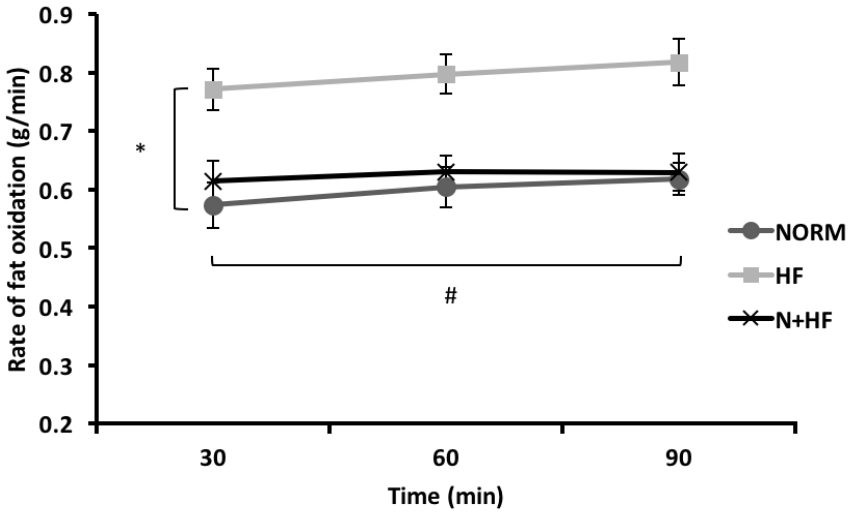

Figure 5-4 Rate of fat oxidation over 90min exercise bout

Data are means with error bars SEM, symbols represent; # main effect of time ($p<0.05$), *significantly different from both NORM and NHF ($p<0.01$).

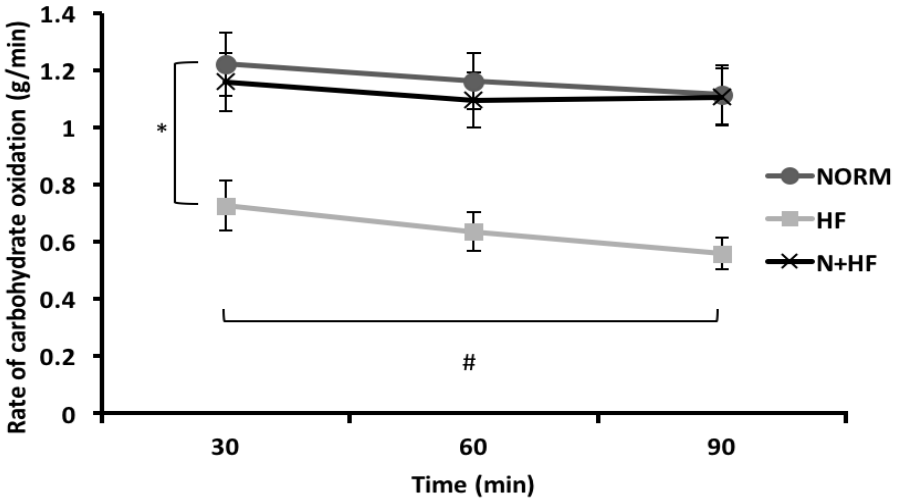

Figure 5-5 Rate of carbohydrate oxidation over 90min exercise bout

Data are means with error bars SEM, symbols represent; # main effect of time ($p<0.05$), *significantly different from both NORM and NHF ($p<0.01$).

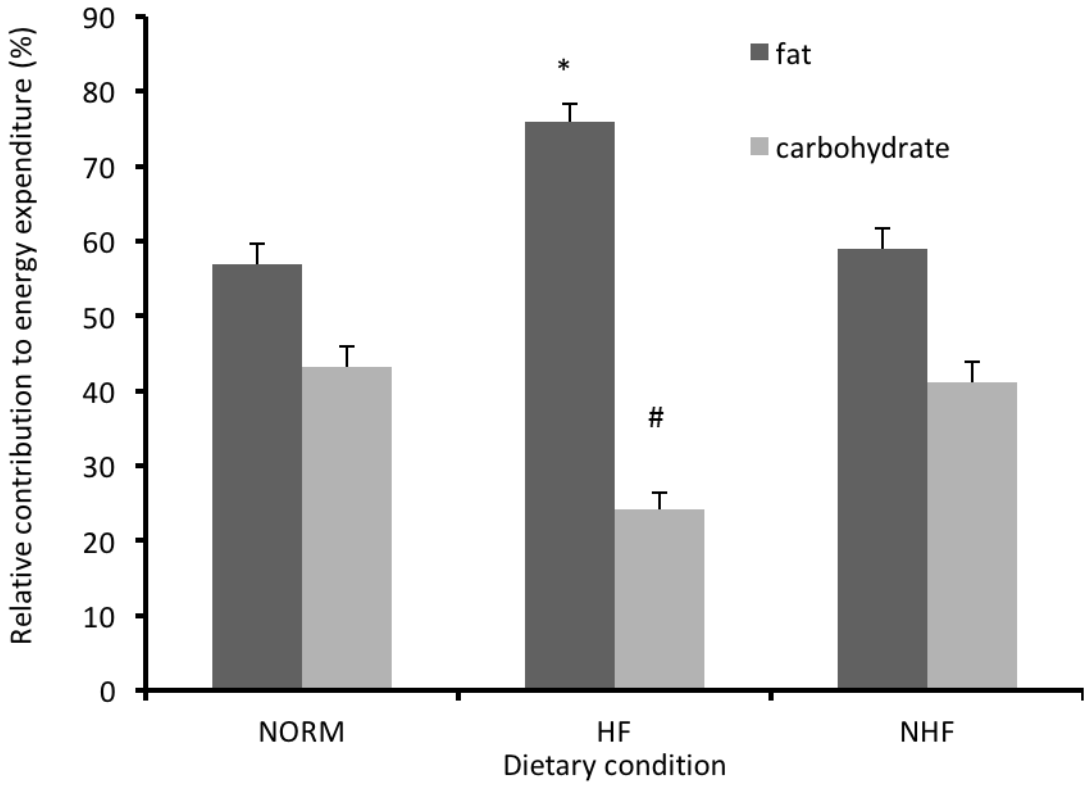

Figure 5-6 Relative contribution of fat and carbohydrate oxidation to energy expenditure

Data are means with error bars SEM, symbols represent significantly different ($p<0.01$), to both NORM and NHF for fat * and # carbohydrate contribution to energy expenditure respectively.

5.3.3.3 Substrate oxidation and contraceptive use

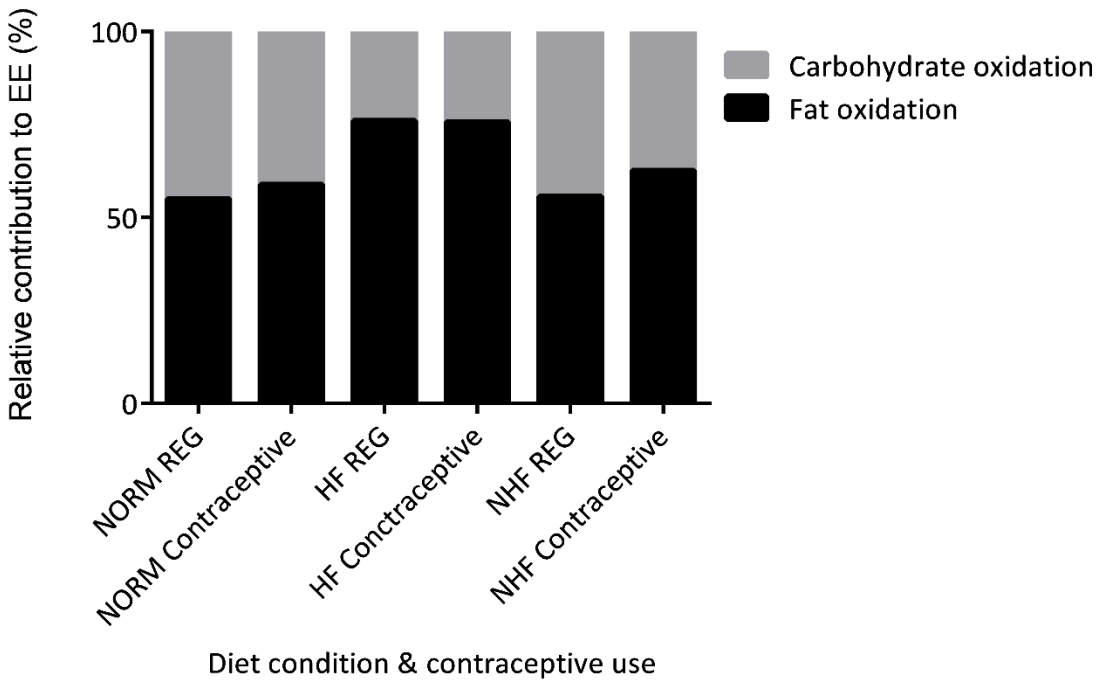

Figure 5-7 Relative contribution (%) to energy expenditure for women regularly menstruating (REG) (*n* 8) and using progesterone contraceptive (*n* 7)

There were no significant differences in rates of substrate oxidation during exercise between regularly menstruating women and women using progesterone only hormonal contraceptive. Figure 5-7 shows the relative contribution of fat and carbohydrate to total energy expenditure (%) across the different dietary conditions separated by contraceptive use.

5.3.4 Blood metabolites

5.3.4.1 Resting values

The resting plasma glucose, lactate and glycerol concentrations, did not significantly differ after the dietary interventions (Table 5-4). The resting serum insulin level in the NORM trial was similar to both the HF and NHF, whereas it was significantly ($p<0.05$) lower in the HF trial compared to the NHF. The calculated HOMA-IR showed a main effect of diet ($P<0.05$) however none of the pairwise comparisons was statistically significant, although there was a trend ($P = 0.07$) for the HF to marginally lower than the NHF. The resting plasma NEFA values did not significantly differ between trials but there was a trend for a main effect of diet ($P=0.07$) with the HF trial appearing greater than the NHF ($P=0.07$). There was a main effect of diet for the total plasma cholesterol levels, which were 9% higher in the HF trial than the NORM ($p<0.01$) with a non-significant 5% difference between the HF the NHF ($p=0.09$). There were no significant differences between the study diets in the LDL -C whilst HDL -C was ~8% ($p<0.05$) lower and TAGs 38% higher ($p<0.01$) following the NORM than either the HF or NHF diets.

The serum sex hormone data demonstrated a wide range of values for both oestrogen and progesterone. Although not as substantial as the regularly menstruating women, variability was still evident within the hormonal contraceptive users Figure 5-8 & Figure 5-9. The resting serum oestrogen and progesterone levels can be seen in Table 5-4 with the median values and individual dot plots demonstrating the intra individual variability and outliers separated by contraceptive use. Typical reference ranges for the early, mid-follicular,

ovulatory and mid-luteal phases of the menstrual cycle can be also be seen in Table 5-5 for a comparison. Regrettably, despite great efforts to test within the mid follicular phase to control for menstrual cycle hormonal fluctuation, it appears the hormonal environment was more varied than originally planned. There were no significant differences in either oestrogen or progesterone concentration between the different diet conditions, when compared with or without contraceptive use as between subjects factor.

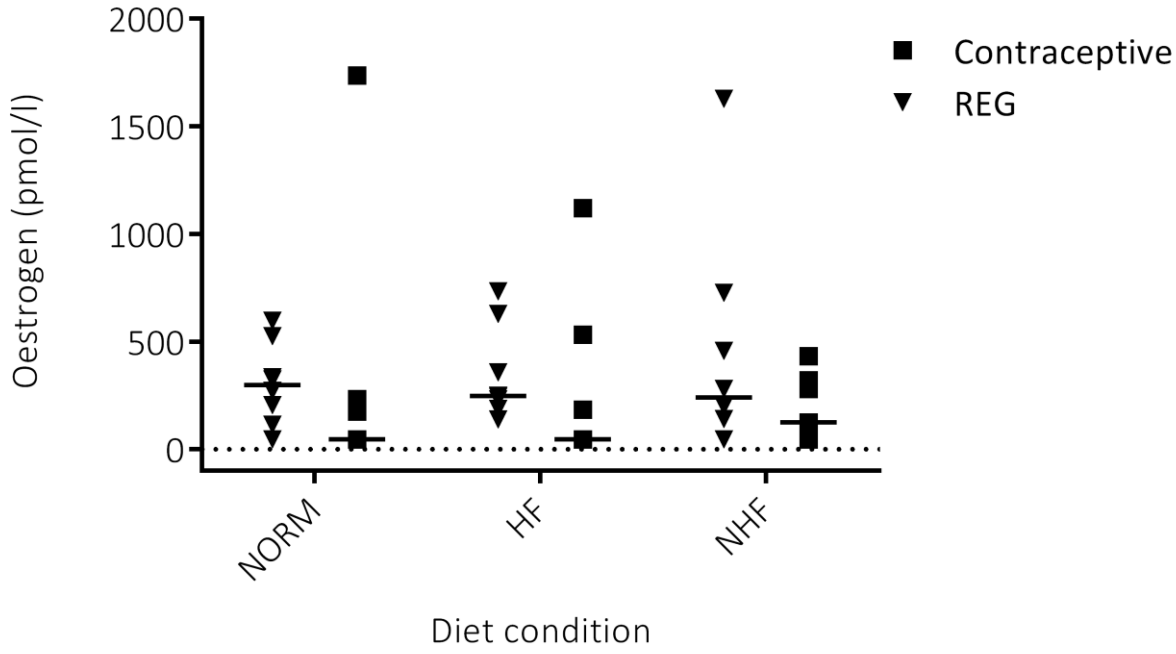

Figure 5-8 The resting serum oestrogen concentration split by contraceptive use

Horizontal bars represent the group median values for resting oestrogen concentration over the different dietary interventions split by groups into non-contraceptive users regularly menstruating (REG) (*n* 8) and those using progesterone only form of contraceptive (Contraceptive) (*n* 7).

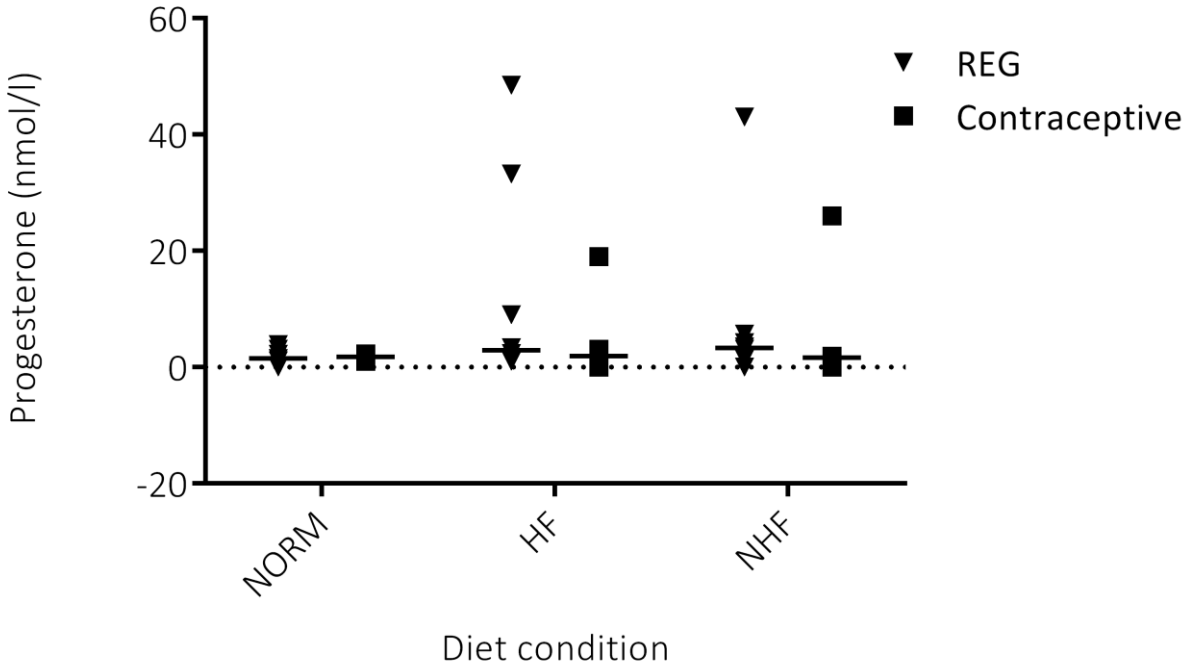

Figure 5-9 The resting serum progesterone concentration split by contraceptive use

Horizontal bars represent the group median values for resting progesterone concentration over the different dietary interventions split by groups into non-contraceptive users regularly menstruating (REG) (*n* 8) and those using progesterone only form of contraceptive (Contraceptive) (*n* 8).

Table 5-4 Resting blood parameters

Parameter	NORM	HF	NHF
Glucose (mmol/l)	5.2 ± 0.5	5.2 ± 0.6	5.3 ± 0.5
NEFA (mmol/l)	0.40 ± 0.20	0.45 ± 0.20	0.24 ± 0.17
Glycerol (µmol/l)	28.5 ± 64.0	36.0 ± 23.5	24.0 ± 53.0
Lactate (mmol/l)	0.70 ± 0.17	0.77 ± 0.18	0.96 ± 0.51
Insulin (µU/ml)	9.0 ± 2.5	8.2 ± 3.7 [c]	9.9 ± 2.7[b]
HOMA-IR	2.1 ± 0.6	1.9 ± 0.9	2.3 ± 0.7
TAG (mmol/l)	0.55 ± 0.17	0.39 ± 0.11[a]	0.41 ± 0.10[a]
Total cholesterol (mmol/l)	4.2 ± 0.6[b]	4.6 ± 0.6[a]	4.4 ± 0.6
LDL (mmol/l)	2.1 ± 0.4	2.3 ± 0.4	2.1 ± 0.4
HDL (mmol/l)	1.8 ± 0.3	2.0 ± 0.3[a]	1.9 ± 0.3[a]
Oestrogen (pmol/l)	205 ± 289	232 ± 487	200 ± 386
Progesterone (nmol/l)	1.5 ± 1.0	2.1 ± 7.7	1.7 ± 2.8

Data are means ± SD for all parameters other than NEFA, glycerol, oestrogen and progesterone which as presented as median ± 25-75 percentiles (*n* = 15). HOMA-IR; Homeostasis model assessment of insulin resistance. Superscript letters represent; [a] significantly different to NORM (P<0.05), [b] significantly different to HF (P<0.01), [c] significantly different to N+HF (P<0.01).

Table 5-5 Reference ranges of oestrogen and progesterone during different phases of the menstrual cycle

	Phase of the menstrual cycle					
Hormone	Early-follicular	Mid-late follicular	Ovulatory	Early-luteal	Mid-luteal	Late-Luteal
Days from peak LH	-15 : -6	-5 : -1	0	+1 : +4	+5 : +9	+10 : +14
Oestrogen (pmol/l)	150 (78-266)	451 (195-1147)	672 (482 -1425)	313 (178 -566)	496 (276 -762)	328 (101-787)
Progesterone (nmol/l)	0.6 (0.3-1.9)	0.6 (0.3-1.6)	2.5 (1.2-4.1)	13.7 (3.2 -39.7)	36.3 (21.2 -54.3)	14.0 (2.0-49.0)

Reference values are median and 95 percentiles in parenthesis taken from (Stricker et al. 2006), LH; Luteinising hormone.

Using the reference ranges from the table above the number of subjects tested in the various different phases of the menstrual cycle can be seen in Table 5-6 separated by those regularly menstruating and those using progesterone only form of contraceptive - demonstrating that more of the regularly menstruating women were tested outside of the follicular phase.

Table 5-6 Frequency of post diet testing by menstrual cycle phase

		Phase of the menstrual cycle					
		Early-follicular	Mid-late follicular	Ovulatory	Early-luteal	Mid-luteal	Late-Luteal
Regularly menstruating	NORM	4	1	2			1
	HF	2	2	1		1	2
	NHF	4		1		1	2
Contraceptive users	NORM	6		1			
	HF	5				1	1
	NHF	5	1		1		

5.3.4.2 During exercise

Figure 5-10 though to Figure 5-18 show the blood metabolite response over the 90minute exercise bout. Throughout exercise the plasma glycerol concentrations were significantly elevated above resting values of 24 – 36 µmol/l and continued to increase over time in all conditions ($p<0.001$) reaching a peak at 90mins, with the HF trial significantly ($p<0.001$) higher than both the NORM and N+HF trial at all exercise time points accumulating in a ~30% greater AUC (Figure 5-11), with no differences between the NORM and N+HF trials. The plasma NEFA concentrations were significantly elevated from resting values (0.24 - 0.45 mmol/l) during exercise and continued to rise as exercise progressed in all trials from 30-

90mins peaking at 381-535 mmol/l ($p<0.01$). Throughout exercise the plasma NEFA concentration was greater in the HF trial than the NHF ($p<0.01$) accumulating in a 38% greater AUC, with a weak trend for NEFAs to be higher (AUC 18% greater) in the HF than the NORM ($p=0.11$). There was no significant difference between the NORM and NHF over the exercise bout, but there was a trend ($p=0.06$) for the AUC to be 24% greater in the NORM than the NHF.

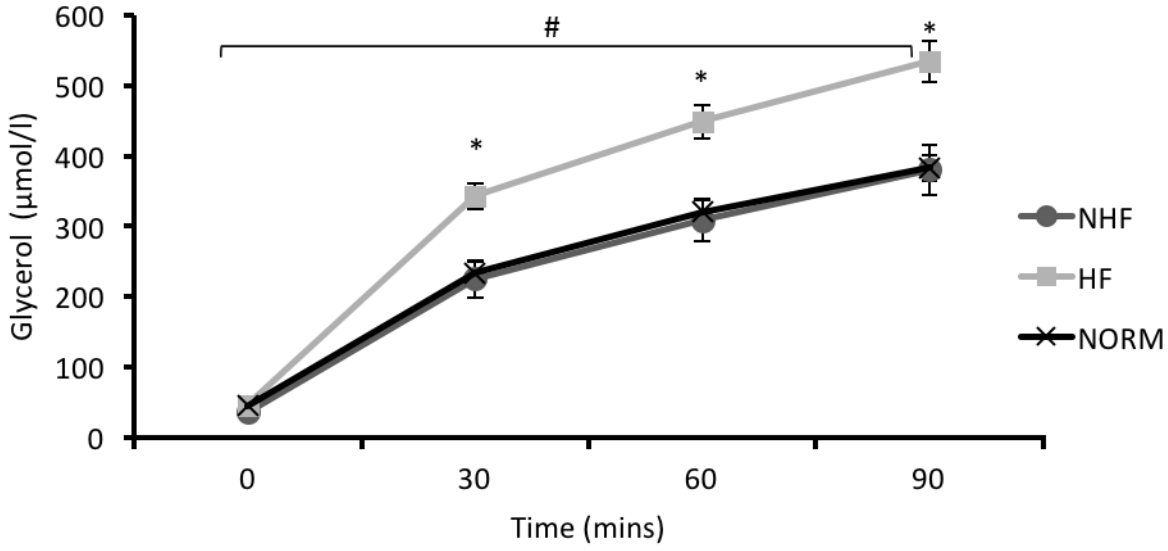

Figure 5-10 Plasma glycerol

Data are means with error bars SEM, symbols represent; # main effect of time ($p<0.001$), *significantly different from NORM and NHF ($p<0.01$).

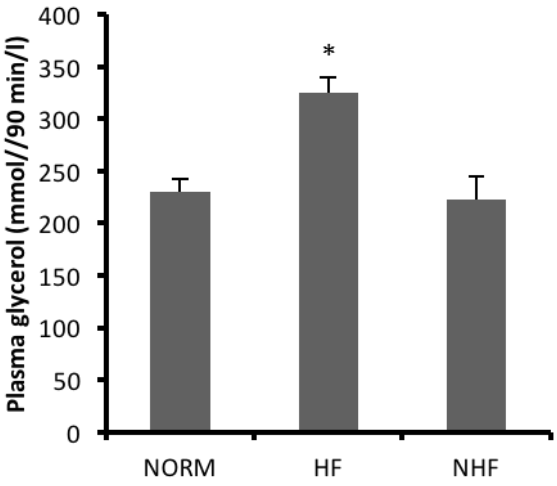

Figure 5-11 Plasma glycerol area under the curve

Data are means with error bars SEM, * represents significantly different from both NORM and NHF ($p<0.01$).

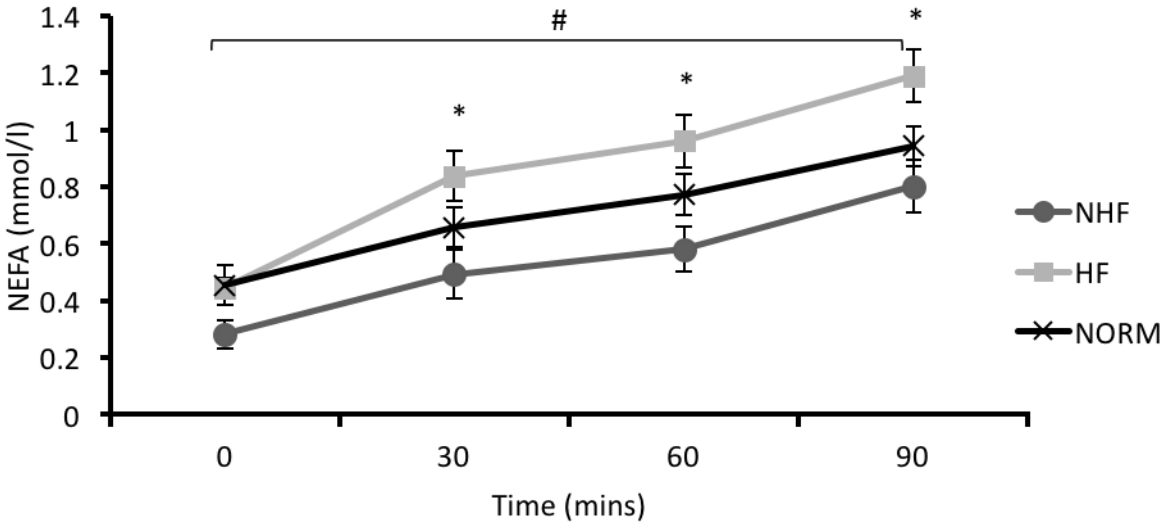

Figure 5-12 Plasma NEFA

Data are means with error bars SEM, Symbols represent; # main effect of time ($p<0.01$), *significantly different between HF and NHF ($p<0.05$).

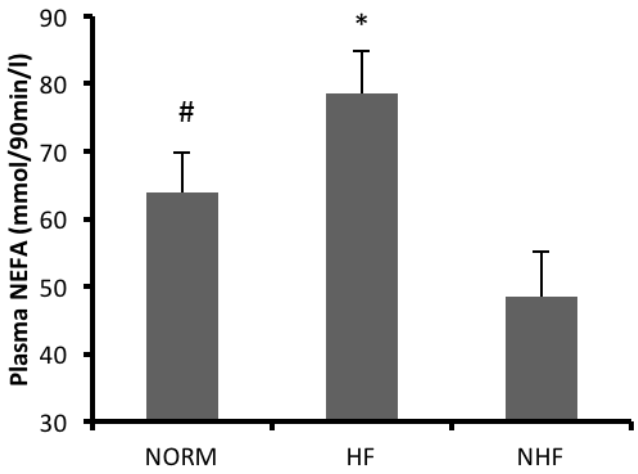

Figure 5-13 Plasma NEFA area under the curve

Data are means with error bars as SEM, * represent significantly different between HF and both NORM and NHF ($p<0.05$), # trend ($p=0.06$) for NORM vs. NHF.

Plasma glucose did not change from rest (5.1 – 5.5mmol/l) in the HF trial, whereas there was a significant increase from rest in both the NORM and NHF trials (5.2 – 5.3mmol/l), with glucose level then remaining stable throughout the exercise bout in all trials (5.4 – 5.8mmol/l). There was a main effect of diet and a diet by time interaction, with the plasma glucose level elevated in the NHF compared to the HF trial at all exercise time points ($p<0.01$), accumulating in a 6% greater AUC ($p<0.05$). The plasma lactate levels during exercise were similar to resting values and remained stable between trials over the course of the exercise bout (0.7-0.82mmol/l) with no significant differences in the AUC. Serum insulin levels showed a significant ($p<0.05$) main effect for time with the concentration declining from resting values of 8.2 – 9.9 µU/ml to 6.7 - 8.0 µU/ml at 60mins, with no differences between the other time points. There were however, no significant differences in serum

insulin concentrations between the study diets, although there was a trend for an effect of diet (p=0.06) with insulin appearing to be lower in the HF diet than the NHF (p=0.07). When summarised using the AUC this difference reached statistical significance, accumulating in a 14% lower AUC in the HF than NHF (p<0.05) but with still no differences compared to the NORM.

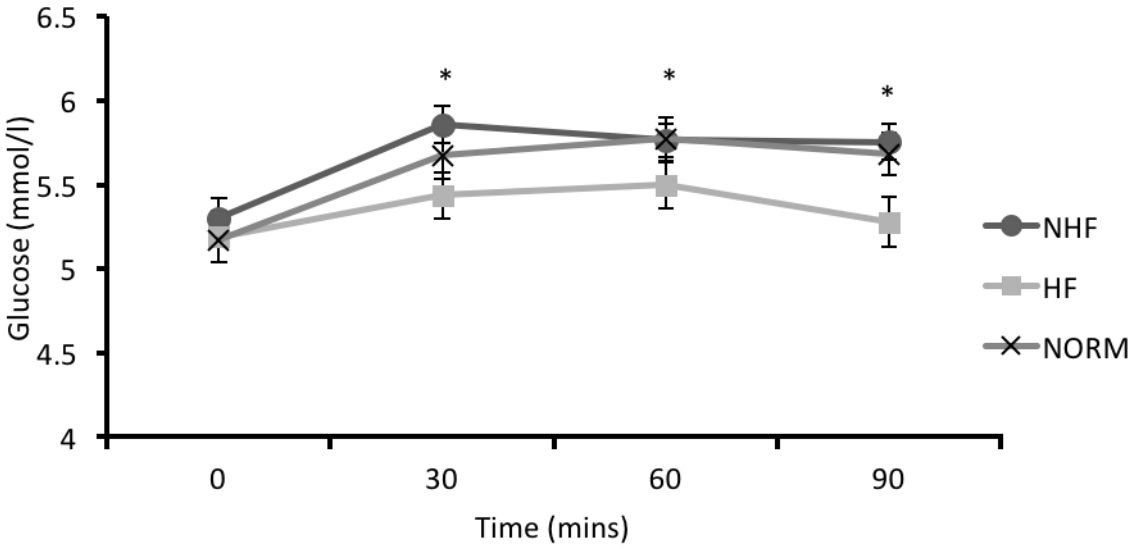

Figure 5-14 Plasma glucose

Data are means with error bars SEM, * symbols represent significant (p<0.05) difference between HF and NHF.

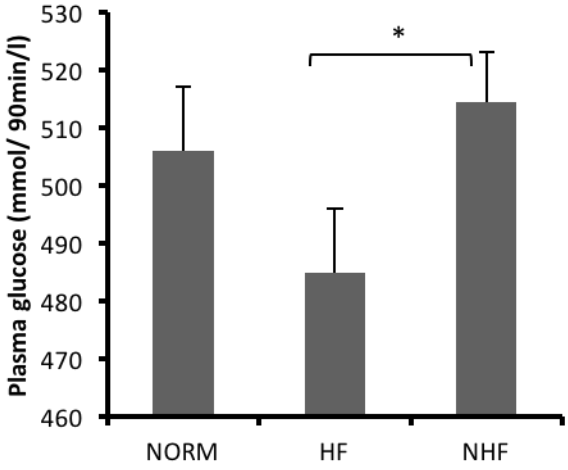

Figure 5-15 Plasma glucose area under the curve

Data are means with error bars SEM, * identifies significant difference ($p<0.05$) between HF and NHF

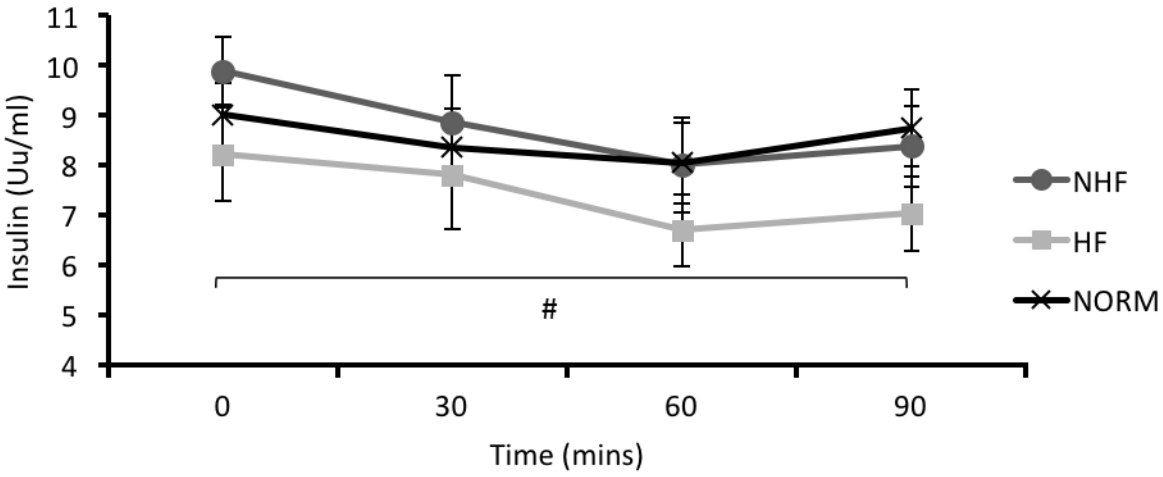

Figure 5-16 Serum Insulin

Data are means with error bars representing the SEM. # represents main effect of time ($p<0.05$) with 60mins lower than 0mins, with no significant differences between trials, although a trend ($p=0.06$) for lower insulin in the HF trial compared to the NHF.

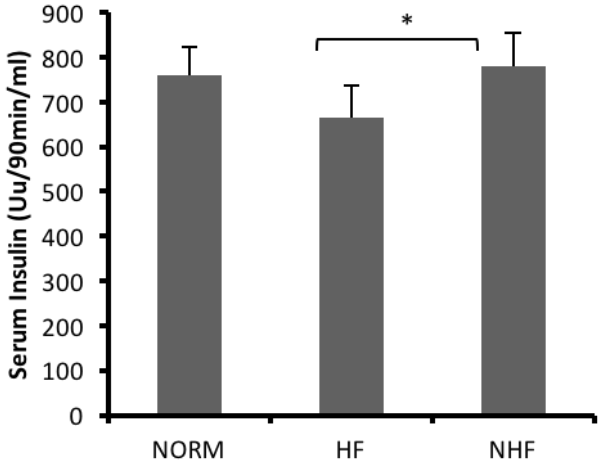

Figure 5-17 Serum Insulin area under the curve

Data are means with error bars representing the SEM. * identifies significant difference ($p<0.05$) between HF and NHF.

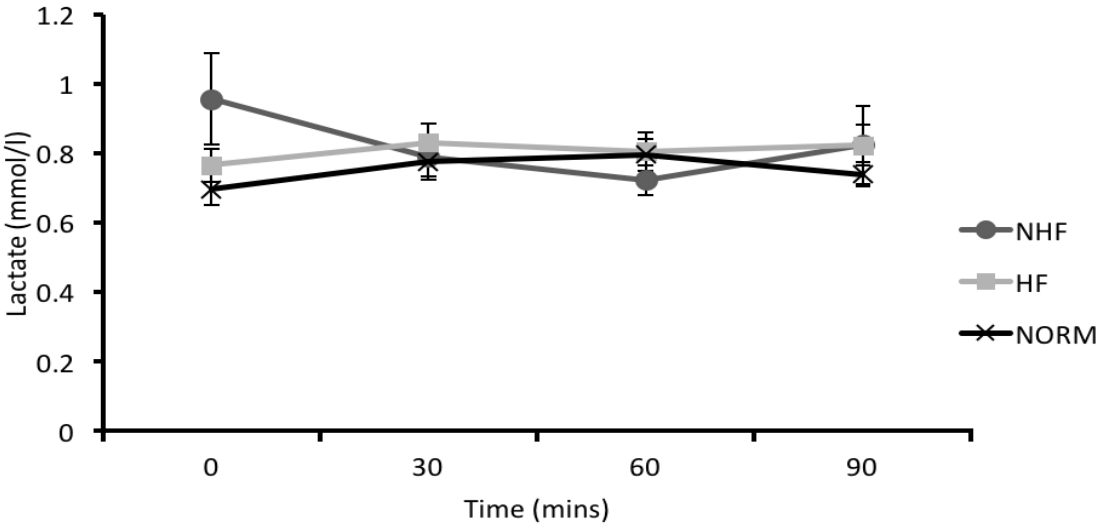

Figure 5-18 Plasma Lactate

Data are means with error bars representing the SEM. No significant differences between trials or over time.

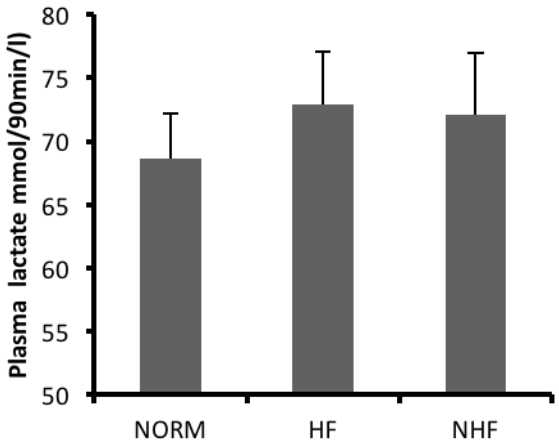

Figure 5-19 Plasma lactate AUC

Data are means with error bars representing the SEM. No significant differences between trials.

5.3.5 Metabolomics

Using an UHPLC-MS metabolomics approach, a total of 217 and 233 metabolites were identified as significantly different across the study diets at rest and at the 90min point of the exercise bout, respectively. After correcting for FDR, the number of significantly different metabolites reduced to 93 at rest and 60 at the 90min point of the exercise bout. Using the FDR corrected data set, at rest, there were 28 significantly different metabolites in the NORM trial vs both the HF and the NHF trial, with this predominantly from the triacylglyceride (21) and diacylglyceride (6) class of metabolites. There were 39 metabolites identified as significantly different in the HF diet vs both the NORM and the NHF trial with these largely belonging to the glycerophospholipid (11) and acyl carnitine (9) class of metabolites. At the 90min point of the exercise bout there were no longer any significant

differences in metabolites when comparing the NORM to both the HF and the NHF, whereas there were 52 significant different metabolites in the HF vs both the NORM and NHF trials, with these mainly belonging to the acyl carnitine (21) or sphingolipids (7) class of metabolites.

Using the non-FDR corrected data set, a total of 153, 152 and 148 metabolites were significantly perturbed (either an increase or decrease) when comparing rest to exercise in the NORM, NHF and HF trial respectively. Figure 5-22 shows the number of significant metabolites from each metabolite class where at least 4 metabolites changed from rest to exercise in at least one dietary condition. The metabolites that significantly changed from rest to exercise mainly belong (>35 individual metabolites in each dietary condition) to the acyl carnitine metabolite class, and 27 individual metabolites from the triglyceride class of metabolites in the NORM trial. Using the FDR corrected data set however, the number of metabolites significantly perturbed from rest to the 90min point of the exercise bout decreased to 70, 51, 81 for the NORM, NHF and the HF respectively. Figure 5-20 & Figure 5-21 have been included to demonstrate the fold change in the two most perturbed metabolite classes, with a fold change <1 indicative of an increase from rest to the 90min time point of the exercise bout and a value >1 a decrease.

Figure 5-20 Change in triacylglyceride metabolite concentration (non-FDR corrected) from rest to the 90min time point of the exercise bout

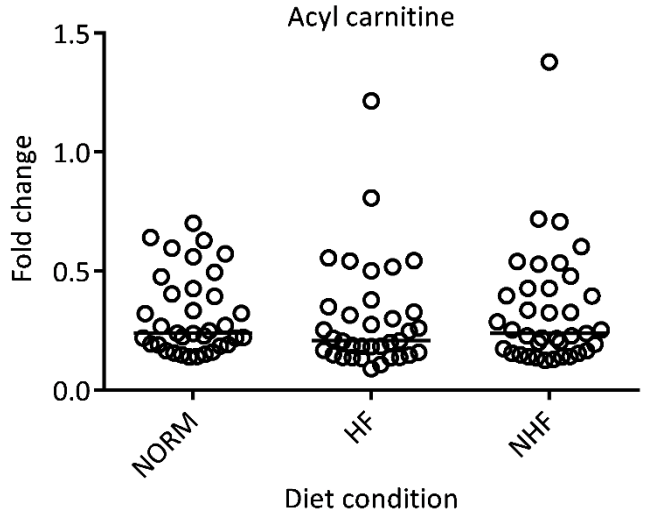

Figure 5-21 Change in acyl carnitine metabolite concentration (non-FDR corrected) from rest to the 90min time point of the exercise bout

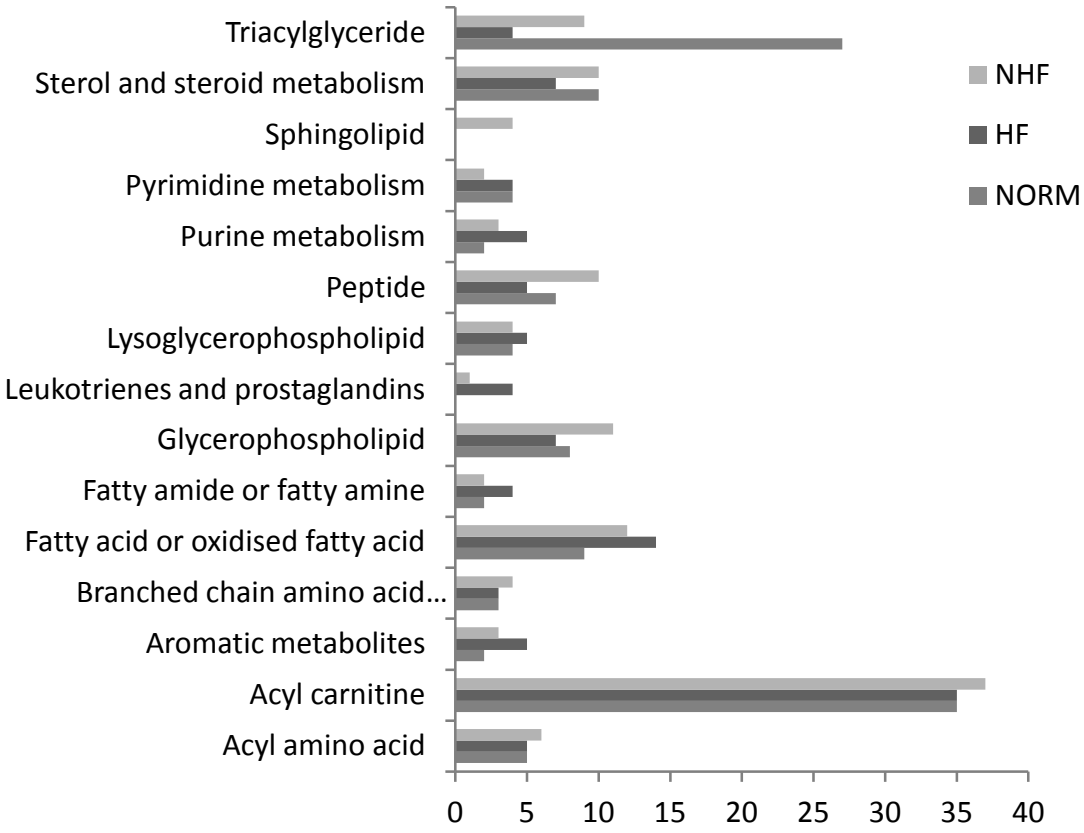

Figure 5-22 Metabolite classes containing > 4 individual metabolites with a statistically significant non FDR corrected (p<0.05) fold change from rest to 90mins of moderate-hard intensity exercise

5.3.6 Time trial

There were no significant differences in the time taken to complete the pre-diet intervention 5km TTs; NORM (1322.5 ± 91.5sec), HF (1325.2 ± 85.0sec) and NHF (1321.9 ± 79.1sec), with the average intra-individual coefficient of variation for these "familiarisation" TTs 2.8 ± 1.8%). There were also no significant differences in the time taken to complete the post diet TTs between any of the dietary interventions; NORM (1328.4 ± 82.7sec), HF (1349.4 ± 75.9sec) and NHF (1332.6 ± 75.9sec), see Figure 5-23, with no order or learning effect

apparent with the time taken for the first (1334.5 ± 85.9sec), second (1336.7 ± 85.0secs) and third (1339.2 ± 85.0secs) post diet TT not significantly different. Nor were there any significant differences between the pre – post diet TTs. There were also no significant differences between the post diets TTs for the pacing strategy employed (Figure 5-24), although a main effect of distance was seen, with the first km ran the slowest and the final km ran the quickest, with no change between km 2-4. Heart rates showed a progressive increase from the first to last km ($p<0.01$) with a similar response seen between the trials although there was a weak trend ($p=0.08$) for the heart rate to be lower in the NORM compared to both HF and NHF which is consistent with the trend seen during the steady state run.

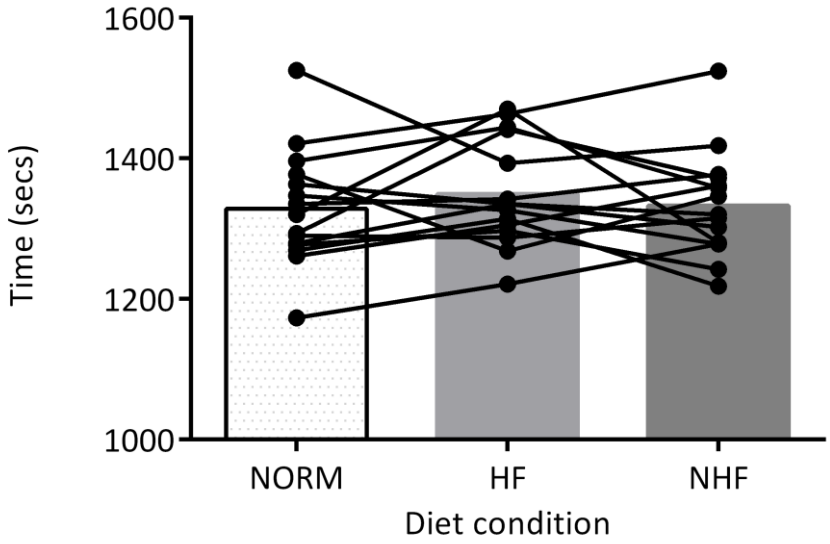

Figure 5-23 Time trial

Bars represent the mean time (secs) taken to complete the 5km TT, with connected points representing individual subjects TT responses to the different dietary condition.

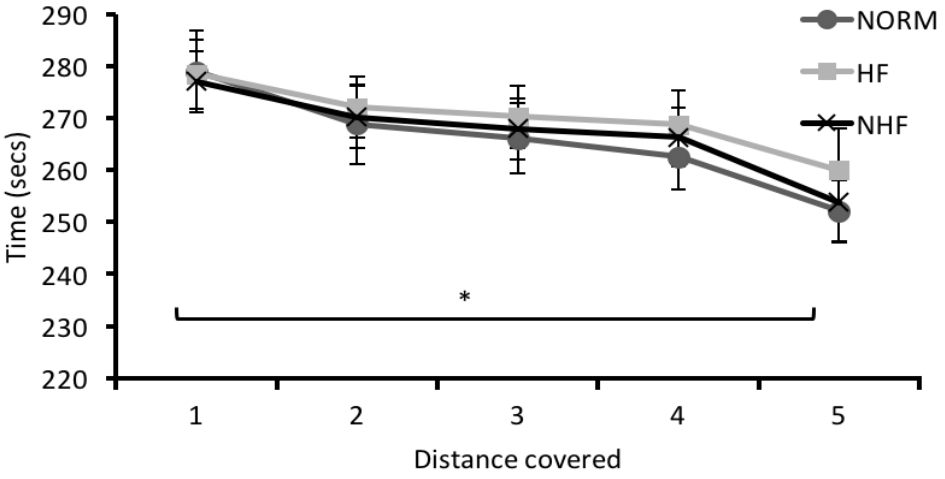

Figure 5-24 Time splits over the 5km TT

Data are means with error bars representing the SEM. * Represents km 1 and 5 significantly different ($p<0.05$) to all other time points.

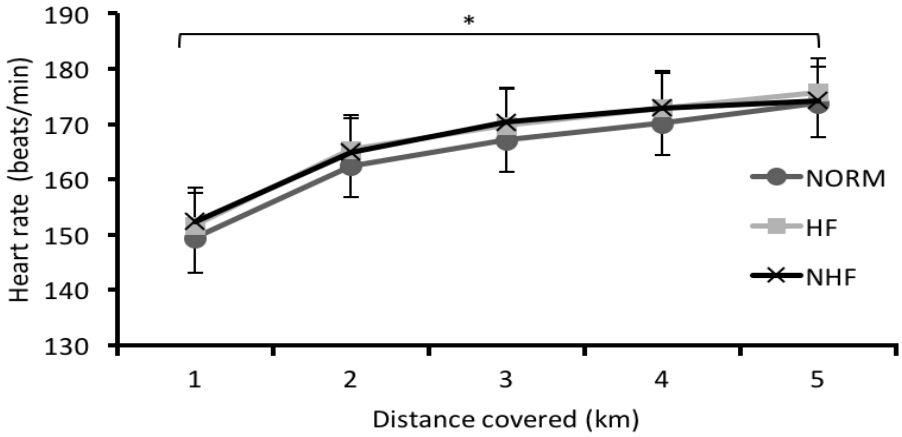

Figure 5-25 Heart response over the 5km TT

Data are means with error bars representing the SEM represents significant main effect ($p<0.01$) for time.

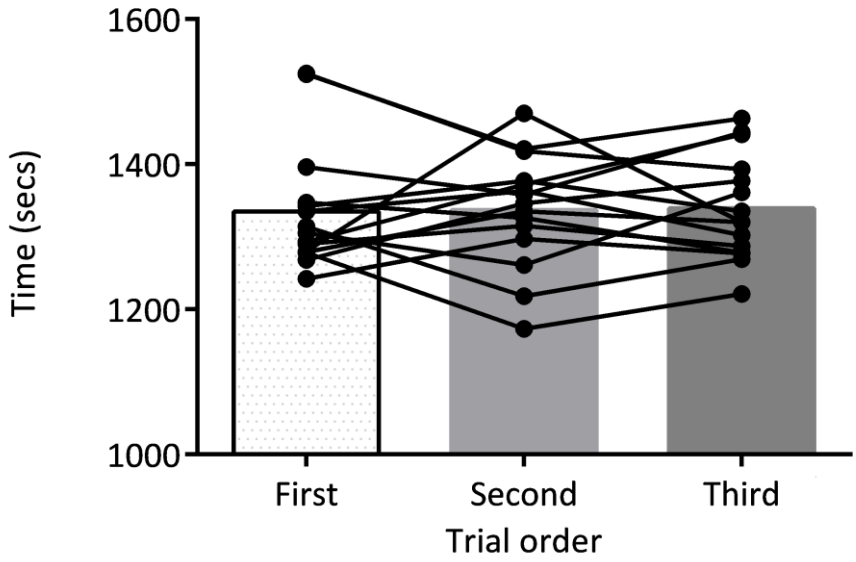

Figure 5-26 Time trial order effect

Bars represent the mean time (secs) taken to complete the 5km TT, with connected points representing individual subjects TT responses in relation to the order trials were ran in.

5.4 Discussion

This investigation aimed to determine whether increasing the amount and availability of fat in the diet independent to carbohydrate intake would affect rates of whole-body substrate oxidation during exercise. A further aim was to better characterise the metabolic response to dietary fat manipulation in both regularly menstruating women and those using progesterone only form of hormonal contraceptives and determine if this translated to differences in exercise performance. In line with the primary aim, the data show that a greater provision of dietary fat per se has little if any influence upon rates of whole body substrate oxidation during exercise. Rather it is the removal or at least restriction of carbohydrate from the diet that appears to be an obligatory step in eliciting dietary induced alterations in whole body substrate oxidation. This study also shows for the first time, that women using progesterone only forms of hormonal contraceptive share a similar metabolic response during exercise *per se* and to alterations in dietary fat content as regularly menstruating non-hormonal contraceptive using women. In addition, within the ranges of macronutrients supplied herein, the total amount and proportion of dietary fat or carbohydrate consumed over five days was shown to have no impact on preloaded 5km endurance performance in women runners.

5.4.1 Substrate oxidation

To our knowledge, this is the first study characterising the metabolic effects during moderate-hard intensity exercise after a HFLC diet consumed for 5 days in trained women.

Akin to previous studies in men, we confirm a substantially (33%) greater contribution of lipid oxidation to energy expenditure during exercise following the HFLC diet than either an iso-energetic NORM diet or an equivalent diet supplemented with extra fat (NHF) (Jansson, Hjemdahl, and Kaijser 1982; Bergstrom et al. 1967; Burke et al. 2000; Decombaz et al. 2013).

In the present study, each dietary intervention commenced with an exercise bout designed to stress and reduce both glycogen and IMTAG stores, with the intention that the ensuing metabolic status of the muscle a reflection of the dietary provision thereafter. Although intramyocellular substrate content was not quantified in the current investigation, based on prior work following similar dietary manipulations, it is reasonable to assume that the myocellular content of glycogen and IMTAG were vastly different following the 3 trials (Fox, Kaufman, and Horowitz 2004; Zehnder et al. 2006). Specifically, that the muscle glycogen content after the HF diet would have been lowest, with the content higher and comparable in both the NORM and NHF due to an equal intake of total carbohydrate. In contrast, the IMTAG content was expected to be lowest after the NORM trial and elevated after the HF and NHF to a similar degree with their comparable intake of total fat. As described in section 2.3 the intramyocellular availability of these substrates are strong determinants of their respective contribution to energy expenditure and overall substrate oxidation during exercise and so likely explain a substantial proportion of the observed difference between the NORM and HF trials. Although, given the duration of the dietary intervention period, it is also likely that the HF diet also induced other adaptations permitting greater uptake, transport and oxidation of plasma sources of fatty acids.

During exercise, the most striking difference between the trials in blood metabolites was that of glycerol and to a lesser extent NEFA. The substantially greater glycerol concentration in the HF than the NORM and NHF trials indicates a much stronger whole body (inclusive of the suggested elevated IMTAG stores) lipolytic response. The somewhat greater NEFA (AUC) availability following the HF trial compared to the NORM (18% p=0.06) and substantially higher than the NHF trial (38% p<0.01), likely contributed towards the greater rates of lipid oxidation in the HF trial. A 5-day a high fat diet has previously been shown to induce greater sympathetic response, promoting lipolysis and elevating plasma NEFA concentration and extraction into the muscle during exercise at 65% $\dot{V}O_2$max (Jansson, Hjemdahl, and Kaijser 1982). This likely compensated for the potentially lower availability of carbohydrate substrates for oxidation with the marginally lower availability of plasma glucose with the assumed substantially limited glycogen availability in the HF trial.

The trend for an overall suppression of plasma NEFA in the NHF compared to the 2 eucalorific trials is in accordance with some prior short term (3-9days) overfeeding studies with samples taken at rest (Samocha-Bonet et al. 2010; Cornier et al. 2007; Brons et al. 2010; Gillberg et al. 2014). However, this is not a consistent finding with others showing short-term lipid overfeeding to not affect or indeed increase resting NEFA concentration (Dirlewanger et al. 2000). In the most similar study to the present, Zehnder *et al* (Zehnder et al. 2006) did not observe a suppression of plasma NEFA concentrations over the course of a 3hr exercise bout after a 1.5-day lipid supplemented diet compared to a high carbohydrate control diet. However, a small reduction in the contribution of plasma NEFA to total fat oxidation was reported following the lipid supplementation (25 ± 3% vs 32 ± 3%). In the

Zehnder *et al* study, the lower availability of plasma NEFA was compensated for with a reciprocal rise in IMTAG use, maintaining a similar overall rate of whole-body fat oxidation. The greater IMTAG storage observed following the 1.5-day fat supplemented diet was unlikely to be directly causing the suppression in plasma NEFA. Rather the suppression of plasma NEFA was more likely a result of the greater insulin response over the first 45mins of exercise, suppressing adipose tissue lipolysis and accentuating the translocation of fatty acid transporters (FAT/CD36) to the plasma membrane for greater plasma NEFA clearance (Luiken et al. 2002).

With only a trend (p=0.07) for marginally lower (~1.2µU/ml) concentrations of the lipolytic suppressive hormone insulin after the HF diet, it seems unlikely that this hormone contributed largely to the greater rates of fat oxidation observed between the N and HF trials. While speculative, other potent regulators of lipolysis such as the catecholamines adrenaline and noradrenaline that were not measured in the current study, but are stimulated to a greater extent after a HF diet could be responsible for the greater whole body lipolytic response seen between HF and NORM (Jansson, Hjemdahl, and Kaijser 1982; Helge, Richter, and Kiens 1996). However, the 14% lower insulin AUC after the HF diet compared to the NHF combined with the substantially lower plasma NEFA in the NHF trial suggests a possible role for insulin in explaining differences in fat oxidation between HF and NHF.

In contrast to when carbohydrate is restricted, simply adding fat to the diet did not augment lipid oxidation during exercise. The blood measurements made suggest a largely similar metabolic response between N and NHF, which may support the substrate oxidation data at least from the perspective of the circulating milieu measured. The similar plasma NEFA concentrations between the NORM and the NHF trials is in agreement with the plasma NEFA data from (Zehnder et al. 2006). Although the catecholamine response was also not measured in the study of Zehnder *et al,* other lipolytic hormones such as growth hormone and cortisol also did not differ between the two dietary conditions suggesting a similar stimulation of whole body lipolysis. Regardless, what the data do allow us to suggest is that with the almost identical rates of substrate oxidation in the NHF and NORM diets and the ~33% greater fat oxidation seen in the HF trial, it appears that dietary carbohydrate availability is primarily responsible for this disparity in substrate oxidation.

The abovementioned findings are consistent with the work of Zehnder and colleagues (Decombaz et al. 2013; Zehnder et al. 2006), although important methodological differences in our study led us to initially hypothesize a different outcome would arise. Firstly, in the aforementioned studies, the much shorter duration of lipid excess (1.5days) was unlikely to induce any major metabolic adaptations that would enhance lipid oxidation other than an increase in IMTAG, with changes in protein content of genes involved in fatty acid transport and oxidation needing closer to 5-days to materialise and take advantage of greater lipid availability. Secondly, the difference in the biological sex of the participants. Women have a greater preponderance to use lipid during exercise, with greater potential IMTAG storage and utilisation than men, and a more favourable hormonal milieu to take advantage of

excess dietary lipid even prior to adapting to a HF diet. Nevertheless, there were no substantial differences reported here, compared to the previous studies that employed men supplemented for a shorter time frame, in the rate of substrate oxidation or in plasma metabolites indicative of carbohydrate or fat metabolism (Decombaz et al. 2013; Zehnder et al. 2006). Our combined findings therefore demonstrate that dietary fat intake is not matched by an up regulation in lipid oxidation unless carbohydrate intake is simultaneously restricted, at least when studied over a 5-day intervention period. This supports the suggestion that carbohydrate availability *per se* is the key determinant of the rate of lipid oxidation during exercise (Sidossis et al. 1996).

These findings though must be viewed in light of the absence of measurements made of the myocellular substrate availability or proposed cellular adaptations to the different dietary interventions. We therefore cannot be certain if any changes in substrate availability or metabolic adaptations occurred following the dietary interventions employed. Although based on prior research following a HFLC diet for an equivalent period it seems likely that the HF trial would have induced at least some of the previously mentioned adaptations, but we are left to speculate somewhat on the adaptations to the NHF trial with insufficient data to the myocellular response to lipid excess without concomitant carbohydrate restriction.

5.4.2 Metabolomics

Using the more holistic approach of metabolomics to explore the independent effect of both the dietary intervention *per se* and of exercise *per se,* was an *a-priori* objective of this study.

Unfortunately, due to delays in processing and analysing the metabolomics data, a more thorough interrogation and exploration of the data has not been possible, however the preliminary analysis allows us to generate some initial insights into the impact of the dietary intervention and exercise on the plasma metabolome.

The snap shot metabolomics data at both rest and at the 90min time point of the exercise bout showed a substantially greater number of sphingolipids, glycerophospholipids and acyl carnitine metabolites to be perturbed following the HF trial than either the NORM or NHF diet. Although we do not have substrate oxidation data at rest, it is possible that this would have matched that of exercise, with greater rates of fat oxidation in the HF trial than the NORM and the NHF trial. Previously acyl carnitines have been suggested as a biomarker of beta oxidation during exercise (Lehmann et al. 2010) . The greater number of acyl carnitine metabolites (in addition to the elevated plasma NEFA data) perturbed in HF as compared to NORM or NHF coupled with the corresponding differences in fat oxidation observed in those trials is thus consistent with the notion of a greater activation and flux through the beta oxidation pathway in the HF trial than the NORM and NHF trials. A further interesting observation from the resting metabolome data was that a large number of triacylglyceride and diacylglyceride class of metabolites were significantly different in the NORM compared to both the NHF and the HF diet. This is in agreement with the resting triglyceride data presented in Table 5-4 and could reflect differences potentially in hepatic triglyceride synthesis in relation to the proportion and/or amount of dietary carbohydrate and fat intake

(Mittendorfer and Sidossis 2001). The change in triglyceride metabolites from rest to exercise however, was similar across the 3 diets as evident in Figure 5-20.

In contrast to the single time point analysis, the response of the metabolome from rest to exercise (Figure 5-22) was remarkably similar between the dietary conditions regarding the number of metabolite classes and the individual number of metabolites that changed. This suggests that similar pathways were perturbed, and to a similar degree in response to the increased metabolic demand from rest to exercise. An example of this is the acyl carnitine data, which while pronounced across all dietary conditions was quite similar in the direction, magnitude and number of metabolites perturbed regardless of dietary intake. This most likely reflects that fat oxidation was activated in all conditions, although the single time point acyl carnitine data perhaps provide more insight into the magnitude of fat oxidation across the diets. One exception with respect to the exercise response was the triglyceride data, which using the non-FDR corrected data showed a substantially greater number of changes in individual triglycerides in the NORM trial compared to the NHF and the HF trials. The significance of this finding is not clear, but perhaps reflects the greater availability and utilisation of plasma triglyceride species in the NORM trial.

Collectively, the metabolomics data suggest there is some discrimination in the metabolite responses similar in relation to the clear differences observed in fat oxidation between HF and the NORM/NHF conditions (i.e., acyl carnitines). Nonetheless, there are clear large exercise effects and possibly diet-exercise interactions that are worthy of further investigation in order to fully understand the significance of the metabolomics investigation.

5.4.3 Selected blood markers of cardiovascular disease risk and insulin sensitivity

The study diets elicited small yet significant changes in the plasma lipid profiles of this cohort of healthy regularly exercising women. After the dietary intervention, no differences were seen in the LDL-C between the trials, with the higher total cholesterol following the HF diet mainly mediated by the 9% greater HDL-C compared to the NORM, which had a substantially (38%) elevated TAG compared to both the HF and the NHF trials. Similar alterations in the plasma lipid profile following either a high fat low carbohydrate or high carbohydrate low fat diet have been reported previously in endurance trained individuals, with elevations of TAG as high as 50% following a diet higher in carbohydrate and more restricted in fat than the present study (Thompson et al. 1984; Larson-Meyer et al. 2008; Brown and Cox 1998; Hamzah et al. 2009). The effect that carbohydrate intake has on TAG however is also related to the *total* amount of dietary fat, with no significant differences seen between the HF and the NHF diets, the extra dietary fat offset the adverse reduction in HDL and increase in TAG seen in the NORM which is similar to 5-days of fat overfeeding (extra 50% EI) in men (Brøns et al. 2009). A meta-analysis from the Institute of Medicine suggest that for every 5% decrease in total fat, TAG levels will increase by 6% and HDL will decrease by 2.2%, this association was only apparent in the present study when the macronutrient change was iso-caloric (IOM 2005). The finding that TAG levels are only reduced when carbohydrate content is reduced is partially supported by prior high fat over feeding interventions, whereby an extra 1275 kcal (94% fat) a day for 5 days had no impact on fasting TAG (Bakker et al. 2014), i.e. the change of fat *per se* had little impact.

The mechanisms whereby a fat restricted, high-carbohydrate diet adversely alter plasma TAG and HDL -C is thought to involve a carbohydrate and insulin induced activation of both sterol regulatory element–binding protein (SREBP-1c) (a transcription factor that regulates triglyceride synthesis) (Horton, Goldstein, and Brown 2002), and through a suppression of LPL activity (Kiens et al. 1987). LPL catalyses the hydrolysis of TAG rich chylomicrons and VLDL-C in the capillary endothelium, and through the actions of cholesterol ester transfer protein, a reciprocal transfer of cholesterol and TAG occurs between these TAG rich lipoproteins and HDL (Davidson 2010). For a more thorough review of the mechanisms and factors responsible for altering the plasma lipid profile please see a comprehensive review by the American Heart Association (Miller et al. 2011).

Although the NORM diet had this adverse effect on the plasma lipid profile, the effects were only marginal regarding implications for the risk of developing cardiovascular disease. For instance when compared to the current recommendations for women without family history / increased risk of coronary heart disease of (Total cholesterol <5mmol/l, LDL < 2mmol/l, HDL >1mmol/l and TAG <1.7mmol/l) (Miller et al. 2011) none of the subjects was outside of these recommended ranges after any of the study diets. Accordingly, when combined with regular exercise over a period of 5 days in healthy pre-menopausal women, diets vastly different in intakes of total fat and carbohydrate appear able to maintain plasma lipids within the recommended ranges.

Although there was a significant main effect of diet on HOMA-IR, the pairwise comparisons did not indicate which trials significantly differed, but suggested a trend (*P* =0.07) for a slight blunting of insulin sensitivity in the NHF trial compared to the HF trial. Using a hyper-insulinaemic euglycaemic clamp, an almost twofold increase in the hepatic insulin resistance index was observed following 5-days of high fat overfeeding in previously healthy men compared to a diet similar to the current study NORM (Brøns et al. 2009). The daily exercise (days 1-4) performed by the volunteers in the present study that can increase insulin sensitivity for hours to days afterwards (Keshel and Coker 2015) may have masked any deleterious effect of the excess fat in the NHF trial on the calculated HOMA-IR.

5.4.4 Hormonal status and progesterone only contraceptive

This study shows for the first time that the metabolic response during moderate-hard intensity exercise following controlled dietary provision and manipulation does not differ in women using a progesterone only form of contraceptive to those that are regularly menstruating. We also saw no differences in the oestrogen or progesterone values between the different diets ($p>0.05$) when the group was considered as a whole or when compared separately by contraceptive use. The literature is mixed regarding the effect of dietary fat intake and endogenous oestrogen concentration (Wu, Pike, and Stram 1999). Two recent cross sectional analyses of >259 regularly menstruating women however, concluded that there was no association between total and/or specific types of dietary fat intake and sex steroid hormone concentrations (Cui et al. 2010; Mumford et al. 2016). Taken alongside the similar sex steroid values between the diet conditions, it was not considered necessary to

account for the hormone status or contraceptive use of the subjects in any subsequent analyses.

The data from the current study suggest it is unlikely that either the progesterone only contraceptives used in this investigation or the circulating oestrogen and progesterone concentrations are responsible for changes in substrate metabolism reported. However, our measurements of both hormones were obtained at rest, whereas both these hormones have been shown to increase to varying degrees during exercise (Jurkowski et al. 1978), and so our resting values may not have reflected the hormone concentration when the substrate oxidation measures were made and so this cannot excluded. Additionally, as seen in Figure 5-8 & Figure 5-9, considerable variability existed in the gonadal hormone concentration, both within and between subjects even with the progesterone only contraceptive group, likely reflecting the athletic population studied who often present with menstrual cycle irregularities (Bonen et al. 1979; De Souza et al. 1998). Table 5-6 shows that 45% of the testing sessions of the regularly menstruating women commenced outside of the expected follicular phase of the menstrual cycle and although this is not uncommon (D'Eon et al. 2002; Casazza et al. 2004) it may confound the interpretation.

5.4.5 Time trial performance

A final objective of this investigation was to delineate the effect of increasing dietary lipid availability on endurance performance in trained women runners. Sample size calculations performed post analysis, using a power of 0.8 with an alpha of 0.05, and the calculated η^2

effect size 0.06, suggest an additional 7 subjects were needed to observe a true effect between the different dietary groups. Nonetheless, from the sample size used, this investigation shows for the first time in women that a HFLC diet, and a fat supplemented diet not restricted in carbohydrate has minimal impact on the performance of a 5km fasted TT. Although the time taken to complete the 5km TT after the HF diet was 1.6% and 1.3% longer than the NORM and N+HF trials respectively, this did not amount to a statistically significant difference, neither was the 0.3% difference between the NORM and NHF trials.

Despite the substantial differences in substrate oxidation rates between the trials, there were no positive or negative effects of a HF diet consumed for 5 days compared to both a normal diet and one supplemented with extra fat. Although it could be argued than a more suitable comparison diet would have been a high carbohydrate diet, an initial aim was to investigate the effects of a diet more typically followed by women runners, and with no difference to the subject's habitual diet our prescribed NORM diet fulfilled this objective. Whilst this inevitably meant the fat intake between the NORM and HF diets were less contrasting than if a HCLF diet had been used, this study still observed vastly greater rates of lipid oxidation during the HF trial, which is inferred as reduced rate of muscle glycogen oxidation, likely through lower availability and/or a diminished capacity to use it (Stellingwerff et al. 2006).

In the current study to ensure no interference with the assessment of performance, no metabolic observations were made that might have offered insights into differences in

metabolism reflective of myocellular substrate depletion during or at the end of the TT. Nonetheless, with similar times taken to complete the TT and no differences between trials in the pacing strategy used, it could be suggested that dietary carbohydrate intake and by inference muscle glycogen content, was not a major determinant of endurance performance in women following 5-days adaptation to a HF diet in the present study.

Although no pre-intervention dietary control was implemented in this study, participants were required to refrain from strenuous exercise the day prior to commencing an intervention diet. In this regards, and with no differences seen in body mass or aerobic fitness at the start of each dietary intervention, the pre-diet "re-familiarisation" TT's provide a useful measure of the reliability of the 5km TT. The average intra-individual CV of 2.8 ± 1.8%, and the average intra-class correlation coefficient 0.87 (95%CI 0.70 – 0.96) is similar to the CV of 1.26 ± 0.45% reported for a 10km run after a similar 90min preload at 65%$\dot{V}O_2$ max suggesting a reasonably good repeatability of the test (Russell et al. 2004). Additionally, the lack of an order effect implies that any potential learning effect was accounted for by the multiple familiarisations / practice TT runs at the start of each trial.

Our trend for a lower heart rate in the NORM trial throughout both the submaximal 90min preload and over the course of the 5km TT despite similar running speeds and times between trials is indicative of a greater catecholamine response to the extra dietary fat in the HF and NHF trials (Jansson, Hjemdahl, and Kaijser 1982; Helge, Richter, and Kiens 1996).

The marginal elevation in heart rate is suggestive of a dietary fat induced greater cardiovascular demand, although this did not transpire into any change in performance.

5.4.6 Body composition

In both the HF and the NORM diets, as intended, the participants were estimated to be in state of energy balance with the prescribed diets closely matching the estimated energy expenditure, whereas the NHF diet induced a state of positive energy balance (~125%). Despite the close matching of intake and expenditure in the NORM and HF diets, there was a significant 0.63 and 1.45kg loss of body mass seen from pre to post diets respectively, with no change in body mass following the NHF diet. The lack of change in body mass following the NHF is similar to other overfeeding studies of this duration and magnitude of caloric excess (Gillberg et al. 2014), with an amplification of either of these parameters likely to induce significant weight gain. The significantly greater body mass loss in the HF trial (than both the NORM and NHF) was likely related to the assumed greater reduction in glycogen stores and the associated storage of the 3-4g of water alongside each molecule of glycogen (Olsson and Saltin 1970). This equally could explain the attenuated loss of weight in the NORM trial where the intake of carbohydrate was not limited to the same extent as the HF diet but with the daily exercise (day 1-4) may still have induced net glycogen degradation.

5.4.7 Conclusion

In endurance-trained women, it is the removal of carbohydrate from the diet that is the decisive component of a high fat (60-65% EI) diet that augments greater rates of whole body lipid oxidation during moderate-hard intensity exercise. The provision of supplemental dietary fat (additional 1.5g/kg BM/day) on the background of a carbohydrate replete diet (6.1g/kg BM/day) does not alter whole body substrate oxidation compared to a diet containing less fat and an equal amount of carbohydrate (6.1g/kg BM/day). Both regularly menstruating women and women using a progesterone only form of hormonal contraceptive had a similar metabolic response during exercise per se and following high fat diets consumed for 5 days, which did not display any deleterious effect on long-term plasma markers of CVD or insulin sensitivity. Finally, the dietary fat and carbohydrate content in the proportion and amounts consumed in the present investigation for a period of 5 days did not substantially impact preloaded (90 min at 65% $\dot{V}O_2$max) 5km running performance performed in the overnight-fasted state.

6 GENERAL DISSCUSSION

The overarching theme of this thesis was to investigate the effect of nutrition on substrate metabolism, metabolic health and endurance exercise performance.

More specifically the aims of the thesis were:

1. To better understand the determinants of the variability in maximal rate of fat oxidation during exercise, with particular reference to the influence of nutrition;
2. To comprehensively characterise the metabolic response in women during moderate-hard intensity exercise following diets high in fat but limited in carbohydrate or high in fat and not limited in carbohydrate. A subsidiary aim was to characterise the metabolic response to dietary fat manipulation in users of a progesterone only form of hormonal contraceptive;

   To investigate the impact of dietary fat manipulation on endurance exercise performance and markers of health in well-trained women.

This section will discuss the key findings providing further comments on limitations and considerations of the findings along with the practical relevance and a concluding summary.

6.1 Inter-individual variation

Using a relatively large cross-sectional study of 305 healthy men and women, Chapter 4 was able to explain ~ 46% of the inter-individual variation in the maximal rate of fat oxidation during exercise, with dietary intake accounting for ~3% of the explained variability. The

other determinants of the variability in MFO were attributed to aerobic capacity, biological sex, fat-mass and self-reported physical activity level.

The substantial 6 fold variability in MFO recorded in Chapter 4 is comparable although slightly greater than that reported by a previous and similarly focused study (Venables, Achten, and Jeukendrup 2005). Indeed, many of the same independent variables identified in Chapter 4 to be key determinants of the variability in substrate oxidation during exercise were the same as the aforementioned study. However, the improvements in methodology and statistical analysis highlighted in Chapter 4 that remove the confounding present in the previous work make the findings in this thesis more robust. Additionally, the analysis in Chapter 4 was also able, for the first time, to associate a modest yet meaningful proportion of the variability in MFO to the intake of carbohydrate and fat. This was directionally consistent with what would be expected from prior dietary manipulation studies (Helge, Richter, and Kiens 1996) whereby a greater intake of fat was associated with a higher MFO with carbohydrate intake showing the opposite association. As well, exploratory analysis indicated that the associations of nutrition with MFO appeared clearer in women than men. As a follow-up to the observations in Chapter 4, the impact of manipulating fat oxidation during exercise through increases in dietary fat intake consistent with the regression analysis was later investigated in women in Chapter 5.

Although an improvement on prior research (Venables, Achten, and Jeukendrup 2005), the work in Chapter 4 still left the majority (54%) of the variance in MFO unaccounted for. This is

likely to be an amalgamation of, as of yet unidentified determinants (e.g. altered single nucleotide polymorphisms in key lipid oxidising regulatory enzymes), random error in measurement, and factors that are known to impact substrate metabolism during exercise that were simply not measured. For instance, the intake and subsequent storage of carbohydrate as muscle glycogen, is known to be one of the main determinants of substrate oxidation during exercise. Based on the work of Goedecke *et al* (Goedecke et al. 2000), it is quite likely that a measure of resting muscle glycogen content in Chapter 4 would have either added to the amount of variability explained by the regression analysis or lessened the importance of carbohydrate intake to the regression analysis by likely sharing some of the explained variance. Indeed, this perhaps explains why carbohydrate intake does not feature in any of the stepwise multiple linear regression models used to predict RER during exercise whereas muscle glycogen content features in all (Goedecke et al. 2000).

A limitation not previously addressed in the study from Chapter 4 was that no insights were gained on either a cellular level or of the circulating metabolites known to influence substrate oxidation during exercise. Both of these measures would likely have contributed to the unexplained variance (Robinson et al. 2016). However, at the onset of the study it was decided for practical and recruitment reasons that measuring the plasma metabolite response during exercise at the same time as the measurement of MFO in such a large sample of individuals would have been unfeasible. As an alternative, a resting urine or single blood sample could more easily have been collected for metabolomics profiling, which could have been combined with a measure of resting substrate oxidation perhaps helping to explain a greater proportion of the variability seen (Goedecke et al. 2000). In this regards a

measurement of resting IMTAG content was only relevant in explaining RER at the exercise intensity (50%W peak) most closely matching the average FatMax of Chapter 4 and would likely have contributed substantially (Goedecke et al. 2000).

6.2 Carbohydrate intake as the key dietary regulator of fat oxidation

As suggested from Chapter 4 and confirmed in Chapter 5, carbohydrate intake seems to be the central dietary factor determining the relative proportion of fat and carbohydrate oxidised during exercise. Using the regression analysis from Chapter 4, carbohydrate intake was the only consistent diet related determinant of MFO when the data set was split and explored separately for both men and women. In addition, in the main regression analysis, compared to the other macronutrients, carbohydrate intake had the largest standardised Beta weights, indicating a greater importance in predicting MFO than either fat or protein intake.

The importance of carbohydrate intake determining substrate oxidation was further highlighted in Chapter 5 where rates of fat oxidation during exercise were only changed when the carbohydrate content of the diet was restricted. The increase in fat intake *per se* only achieved an elevation in the rate of fat oxidation when carbohydrate intake was also limited. This is the first study to demonstrate this following a period of dietary change assumed to be of sufficient duration to elicit dietary fat induced adaptations. During exercise, under these conditions of restricted carbohydrate availability the increase in energy demand must be met by an increase in the available substrate – lipid. Under

conditions of ample carbohydrate availability, the increase in energy demand during exercise is met by oxidation of the available substrates – carbohydrate and fat. The disparity between fat availability and fat oxidation when carbohydrate intake is not restricted (seen in the NHF diet) suggests that the presence of carbohydrate negates the need to oxidise the available lipid. This has been described previously as a reverse-Randle cycle (Sidossis and Wolfe 1996), whereby despite the greater availability of fat, which in the original concept of Randle (Randle et al. 1963) would down-regulate carbohydrate oxidation, it appears that during exercise the availability of carbohydrate acts as a brake on the oxidation of fat even when lipid availability is plentiful.

6.3 Menstrual phase and hormonal contraceptives

In Chapter 4 using a questionnaire to document hormonal contraceptive use and a crude calendar based approach to estimate the phase of the menstrual cycle that MFO was determined in, we observed; firstly, that there was no significant difference ($p=0.07$) in the MFO between regularly menstruating women in the follicular compared to luteal phase of the menstrual cycle. Secondly, we observed that those using a hormonal form of contraceptive, irrespective of the type, had a significantly higher MFO than regularly menstruating non-hormonal contraceptive users. Given the oestrogen suppressive effect of PROGEST (Ruan, Seeger, and Mueck 2012; Mäkäräinen et al. 1998) it was surprising to observe greater rates of fat oxidation in this group compared to the regularly menstruating group and to have an equal rate as the women using a combined (exogenous oestrogen) form of contraceptive.

Chapter 5 presented the opportunity to test the observation of greater lipid oxidation when using PROGEST and to better characterise substrate metabolism in these women compared to regularly menstruating women during exercise in response to high fat dietary manipulation. The women using PROGEST were well matched to the regularly menstruating women with no significant differences seen in age, weight, body composition, aerobic capacity or habitual diet. In contrast to the observations in Chapter 4, using the stronger study design in Chapter 5 (parallel, matched groups vs. cross-sectional observation) we report for the first time, that there are no significant differences in substrate oxidation during exercise, or differences in markers of carbohydrate and fat metabolism in the blood at rest or during exercise following what might considered a normal-balanced diet or in response to dietary fat manipulation between PROGEST users and regularly menstruating women.

In Chapter 5, neither the sex hormone concentrations nor the use of a PROGEST contraceptive, were found to statistically influence the outcomes in respect of substrate metabolism during exercise. However, a somewhat unexpected observation was the large variability in sex hormone concentrations even within the PROGEST users. Similar variations in sex hormone concentrations despite efforts to control for the phase of the menstrual cycle have however previously been reported (Casazza et al. 2004) possibly reflecting the pulsatile nature of their release for instance progesterone can fluctuate >17 fold within an hour (Filicori, Butler, and Crowley Jr 1984). Furthermore, despite PROGESTs suppressive

effect, ovarian activity and growth is still known to be present (albeit of a lesser absolute magnitude) and so hormone concentration can still fluctuate in this population (Mäkäräinen et al. 1998; Croxatto and Mäkäräinen 1998). In future studies, if the outcome is expected to be strongly influenced by the concentration of the sex steroids then this is something worthy of consideration, with recommendations provided in a recent article (Stachenfeld and Taylor 2014).

6.4 Exercise Performance

In Chapter 5, we observed no significant effect a HF diet consumed for 5 days compared to both a normal diet and one supplemented with extra fat on the time taken to complete a 5km TT. The vastly lower rates of carbohydrate oxidation in the HF trial were inferred to mean there was a reduced rate of glycogen oxidation compared the NORM and NHF trials, either through reduced availability of glycogen or the capacity to utilise it (Stellingwerff et al. 2006; Bergstrom et al. 1967). Nevertheless, the similar pacing strategy and overall time taken to complete the TT compared to the two trials with presumably greater glycogen availability, suggest that glycogen availability was not a major determinant of endurance performance in women in the exercise model used. It could be argued that the 90min preload and the 5km performance measure was not either intense enough or long enough to fully stress muscle glycogen stores and so this did not limit exercise performance. Indeed there were no clear differences in performance or whole body substrate oxidation following the NORM and NHF trial with assumed equal availability of glycogen, which is in accordance with respect to both substrate oxidation and similarity in exercise performance reported

after a similar but higher carbohydrate diet for 1.5days (Decombaz et al. 2013; Zehnder et al. 2006). Although we saw no difference using a measure of performance, had we chosen a measure of exercise capacity, then perhaps we would have truly stressed the glycogen content and seen similar positive effects to higher dietary carbohydrate intake and availability (O'Keeffe et al. 1989; Bergstrom et al. 1967).

It is also possible that we have a type 2 error, reflecting the notoriously difficult objective of measuring exercise performance or capacity in the laboratory with the reliability of the tests often used insufficiently sensitive to detect a real or meaningful change in performance (Currell and Jeukendrup 2008). Knowing that the determinants of exercise performance are complex and multi-factorial, particularly in endurance events where pacing and the perception of effort can be just as important in determining performance outcome as substrate provision (Hampson et al. 2001), and so other often uncontrolled factors could be playing a part (Halperin, Pyne, and Martin 2015).

6.5 Health perspective and practical implications

Evidence suggests that a low capacity to oxidise fat is an important determinant in the development of obesity and overall metabolic health. For instance, a high resting respiratory quotient (RQ) which is indicative of low relative rates of fat oxidation, is predictive of future weight gain (Zurlo et al. 1990; Marra et al. 2004; Shook et al. 2015; Ellis et al. 2010), the development and progression of NIDDM, hypertension and atherosclerosis (Montalcini et al.

2016; Ferro et al. 2013; Montalcini et al. 2012). As well, a strong association exists between the MFO achieved during exercise and both the rate of fat oxidation measured over a 24hr period in a whole room respiratory chamber and insulin sensitivity (Robinson et al. 2015). Thus the better understanding of the determinants of MFO observed in chapter 4 may help both metabolic health through exercise prescription and body composition improvements / maintenance (Venables and Jeukendrup 2008).

With the consistent finding between Chapter 4 and the work of Venables *et al* (Venables, Achten, and Jeukendrup 2005), aerobic capacity and physical activity appear to be the most robust modifiable determinants of MFO that could be the focus of public health interventions aimed at improving metabolic health and body composition. Whilst dietary intake is also a modifiable factor, the substantially greater Beta coefficient weighting in the regression analysis for aerobic capacity and physical activity indicate that these would likely induce greater effects on MFO and by inference metabolic health. Although the cross sectional study design that identified these associations does not permit causality and firm conclusions to be drawn.

From the sex specific regression analysis from chapter 4 in women, it would be predicted that a 150g/day increase in the total amount of fat in the diet whilst keeping all other determinants of MFO constant (aerobic capacity, physical activity level, body composition, carbohydrate and protein intake), would be associated with an increase MFO of 0.15 g/min. This prediction however, was not substantiated in Chapter 5 with the extra dietary fat

provided in the NHF trial not changing whole body fat oxidation compared to the NORM trial. As described previously, this implies that rather than a push to oxidise more fat during periods of high fat availability the carbohydrate brake must also be removed to augment fat oxidation (Sidossis and Wolfe 1996).

The lipid overfeeding model used in Chapter 5 although providing insights into the metabolic effect during exercise to short term periods of over-consumption, is not a model to consider from the perspective of inducing an increase in fat oxidation to improve metabolic health or body composition. In this regard, a state of dietary induced negative energy balance would be sought with the composition of the reduced macronutrient intake needing to be fat. Recent data suggest that under dietary induced negative energy balance conditions, a restriction of dietary fat leads to a greater body fat mass loss than an isocaloric restriction of carbohydrate. This is despite the restriction of carbohydrate augmenting a greater rate of fat oxidation, the overall net fat loss after accounting for fat intake is greater (Hall et al. 2015).

In Chapter 5, minimal adverse health effects were seen following the acute (5day) lipid overfeeding on markers of CVD risk, body composition and insulin sensitivity. This is suggestive that short periods of lipid overfeeding are not overly detrimental to health. It is quite likely that the regular exercise and physical activity of the women studied in Chapter 5 offset to some extent the expected deleterious effects of the excess dietary fat, thus when overconsumption is likely, staying as physically active as possible may offer some metabolic protection (Walhin et al. 2013). However, had the diet continued for a period longer than

5days, then the positive fat and energy balance would undoubtedly have led to greater body fat accumulation which is an independent risk factor for metabolic disorders such as Type 2 diabetes. It would seem sensible to suggest that periods of overconsumption must be met with a similar increase in energy expenditure in order to mitigate against body fat accumulation.

6.6 Limitations

The limitations of methods used *per se* or in the study designs have been addressed throughout the thesis as they became relevant, however two further potential considerations not previously discussed are addressed below. In Chapter 5 it could be argued that the Actiheart activity monitors that were used to estimate EE, are not valid for this purpose during periods of over feeding due to the potential impact overfeeding has on resting heart rate (Walhin et al. 2013). Indeed, 6 weeks of overfeeding by 150%EI was previously shown to increase resting heart rate by 5bpm (Norgan and Durnin 1980) and so this may have resulted in small increase in calculated EE in the current investigation as may well of the extra fat in the HF trial (Helge, Richter, and Kiens 1996). However, this is unlikely given that the branched chain equation used by the Actiheart software prioritises activity counts rather than heart rate when at low physical activity levels that occupy the majority of the time. Furthermore, overestimation of energy expenditure would have been offset by a methodological oversight of not accounting for potential dietary induced changes to diet induced thermogenesis (DIT). Although only the smallest component of total energy expenditure, DIT is influenced by the macronutrient composition of the diet, with a

macronutrient hierarchy in the magnitude of DIT, ranging from 20-30% for protein, 5-10% for carbohydrate and 1-3% for fat (Westerterp 2004). The EI and EE calculated for each trial did not account for these differences, rather the standard mixed diet fixed factor of 10% EI was used (Westerterp 2004; Tappy 1996). Thus with both the HF and the NHF diet the EE through DIT would likely have been less than 10%. DIT was not however measured, and so with the small overall contribution to total EE, and the inter-individual variation in DIT it was not individually accounted for.

In Chapter 5, no attempts were made to control the timing of meals / snacks in relation to exercise training sessions over the dietary manipulation period. Recent work however (Marquet et al. 2016) suggests that nutrient availability independent of macronutrient composition can augment or blunt training induced cellular adaptations that later impact substrate oxidation and exercise performance and thus should have been controlled. Moreover, it could also be argued that the 30% excess fat (~kcal700 daily) provided in the NHF trial in Chapter 5 was not sufficient lipid surplus to perturb homeostasis and substantially induce metabolic adaptations to a level detectable in an active cohort of women. However, from a practical perspective, subject compliance would be a major factor in achieving greater than a 30% EI in a cohort of individuals with notoriously restrictive eating habits.

6.7 Future research

Considering that the majority of the inter-individual variability in MFO is unaccounted for, this should be a focus of future research. Utilising substantially larger sample sizes than

those used in Chapter 4, future work should use multiple omics based approaches, with the aim of identifying currently unknown factors regulating substrate oxidation during exercise. This should be combined with measures already known to impact substrate oxidation such as the determinants identified in this thesis but to also factors not measured here but already thought to be important, such as the myocellular substrate availability. With the greater understanding yielded from this combined approach, a better tailoring and individualised prescription of ways to augment lipid oxidation to improve metabolic health could be provided.

Following on from the augmented rates of fat oxidation in women to the HFLC diet in Chapter 5, future research should determine if the cellular adaptations to this intervention are a) similar to men, and b) if they persist after carbohydrate restoration, c) if this elicits improvements in endurance performance.

Despite showing no differences in substrate oxidation between regularly menstruating women and those using a PROGEST only form of contraceptive, the work in animal models suggests a potential for a down-regulation of lipid oxidation. The many metabolic differences seen between rodents and humans require future pharmacological longitudinal based approaches in human, measuring at the cellular level, pre and post intervention to confirm or refute whether the effect seen in rodents is translatable to humans.

6.8 Conclusions

This thesis demonstrated that there is substantial inter-individual variation in the capacity to oxidise fat whilst physically active. Just under half of this variability was explained by aerobic capacity, self-reported physical activity, biological sex, body composition and the dietary intake of fat and carbohydrate. The contribution of diet to the explained variability was modest at ~3%, with the intake of fat associated with an increase in MFO and the opposite relationship observed for carbohydrate intake. Further research utilising a variety of different "omic" based approaches and large cohorts are likely required to delineate the remaining unexplained variance. This thesis also showed that despite initial data that might have suggested the contrary, regularly menstruating women and women using a progesterone only form of hormonal contraceptive exhibited a similar metabolic response during moderate-hard intensity exercise following normal and high fat diets. A major finding of the present thesis was that augmented rates of fat oxidation during exercise following 5 days of high fat intake are reliant on the simultaneous or instead restriction of carbohydrate from the diet, with no substantial difference seen in substrate oxidation to increased fat availability *per se*. Although no differences on substrate oxidation were apparent on a whole body level after this dietary manipulation, confirmation of no changes on a cellular level is worthy of follow up. Finally, in trained women runners, a high fat low carbohydrate diet and a fat supplemented diet not restricted in carbohydrate had minimal impact on surrogate markers of metabolic health or the performance of a pre-loaded (90 min at 65% $\dot{V}O_2$max) 5km time-trial performed in the overnight-fasted state.

7 APPENDIX

7.1 Metabolic simulator output

Oxycon Pro: 808776	Expected	Achieved
Breathing frequency: 20 (l/min)	2.00 ± 0.05	2.01
$\dot{V}O_2$(L/min)	1.009 ± 0.05	1.036
$\dot{V}CO_2$ (L/min)	1.020 ± 0.05	1.054
RER	1.011 ± 0.04	1.02
Breathing frequency: 40 (l/min	2.00 ± 0.05	2.03
$\dot{V}O_2$ (L/min)	2.008 ± 0.08	2.035
$\dot{V}CO_2$ (L/min)	2.029 ±0.08	2.069
RER	1.010 ± 0.04	1.02
Breathing frequency: 60 (l/min)	2.00 ± 0.05	2.04
$\dot{V}O_2$(L/min)	3.030 ± 0.115	3.101
$\dot{V}CO_2$ (L/min)	3.062 ± 0.115	3.111
RER	1.011 ± 0.04	1.00
Breathing frequency: 80 (l/min)	2.00 ± 0.05	2.04
$\dot{V}O_2$(L/min)	4.039 ± 0.15	4.140
$\dot{V}CO_2$ (L/min)	4.082 ± 0.15	4.070
RER	1.011 ± 0.04	0.99

7.2 Food Diary

PLEASE READ THROUGH THESE PAGES BEFORE STARTING YOUR DIARY

We would like you to keep this diary of everything you eat and drink over 4 consecutive days.

Please include all food consumed at home and outside the home e.g. work, college or restaurants. It is very important that you do not change what you normally eat and drink just because you are keeping this record. **Please eat as you normally would do.**

Day and Date

Please circle the study day (1,2,3,4) and write the date at the top of the page each time you start a new day of recording.

Time Slots

Please note the time of each eating occasion into the space provided.

What did you eat?

Please describe the foods you eat in as much **detail** as possible. Be as **specific** as you can. The example day shows the level of detail needed such as –

Cooking methods: (fried, grilled, baked, micro-waved etc).

Any additions (sugar/sweeteners, sauces, pepper, salt, mustard, etc).

Type and amount of fat / oil used for cooking, e.g. teaspoon peanut oil, 15g butter.

What brands, e.g. Kellogg's corn flakes, places food bought, fresh, frozen, tinned, raw.

What variety – e.g. semi skimmed milk, low fat pro-biotic yogurt, reduced salt, reduced sugar?

Whether soft drinks were low calorie (diet) or decaffeinated?

Were fruit juices UHT, pasteurised or freshly squeezed, made from concentrate

Products such as cheese, fish and meat were they smoked or not

Meats – what part of the animal? Chicken breast? Legs? Wings? back bacon, streaky bacon, extra lean beef mince, value beef mince.

E.g. a cheese sandwich is really 3 foods – Bread, Margarine and Cheese. What type / amount of bread? Type / amount of spread? Type / amount of cheese?

Remember to record all snacks and drinks throughout the day.

Portion sizes

Please use the kitchen weighing scales provided to **weigh every item** of food / drink consumed – Remember to Zero after each item of food, and check it's in grams. Eat the amount you would normally eat – don't use the scales to determine when to stop!

On **rare occasions** when weighing is not possible, food quantities can be described using:

• household measures, e.g. one teaspoon (tsp) of sugar, two thick slices of bread, 4 tablespoons (tbsp) of peas, ½ cup of gravy, large portion of takeaway chips. Be careful when describing amounts in spoons that you are referring to the correct spoon size.

• use weights from labels, e.g. 4oz steak, 420g tin of baked beans, 125g pot of Yoghurt – but only if everything is eaten – did you eat the fat /skin **/ bones?**

• number of items, e.g. 4 fish fingers, 1 Rich Tea biscuit, 1 king size mars bar

With foods such as fruit, remember to record the weight of the skin/core/stone in the weight leftover column

For drinks, quantity should be described using **weights**:

• On rare occasions when weighing isn't possible, use the size of glass, cup etc (e.g. large glass) or the volume (e.g. 300ml, 1 pint).

• use volumes from labels (e.g. 330ml can of fizzy drink, 500ml strawberry Innocent smoothie).

We would like to know the amount that was actually eaten which means taking into account leftovers. You can do this in two ways:

1. Record what was served and note what was not eaten e.g. 30g of peas, only 12g eaten; 1 Weetabix, ate ½

2. Only record the amount actually eaten i.e. 18g of peas; ½ Weetabix

Homemade dishes

If you have eaten any homemade dishes e.g. chicken casserole, please record the name of the recipe, ingredients with amounts (including water or other fluids) for the whole recipe, the number of people the recipe serves, and the cooking methods used. Write this down in the recipe section at the end of the diary. Record how much of the whole recipe you have eaten in the portion size column.

Take-aways and eating out

If you have eaten a take-aways or eaten dishes not prepared at home such as at a restaurant or a friend's house, please record as much detail about the ingredients as you can e.g. vegetable curry containing chickpeas, aubergine, onion and tomato. Please also record the name of the restaurant, takeaway, and the name of the meal, e.g. Domino's pizza, 16inch meat feast, thin base.

Brand name

Please note the brand name (if known). Most packed foods will list a brand name, e.g. Bird's eye, Hovis, or Supermarket own brands. For ready-made meals or for less well known brands, please keep the packet's nutritional information in the bag provided.

Supplements / Medications

Please also provide information about any supplements you took. Please record the brand name, full name of supplement, strength and the amount taken should be recorded, e.g. Maximuscle cyclone powder – 40g (2 scoops), Holland and Barret Cod Liver Oil and Glucosamine Capsules (500mg) – 1 capsule.

If you take any new medicines or stop taking ones we know about please record it here.

Was it a typical day?

After each day of recording please tell us whether this was a typical day or whether there were any reasons why you ate and drank more or less than usual. E.g. Drank 4 pints of Guinness as it was St Patricks day, day 2 ate very little as not feeling well.

When to fill in the diary

Please record your eating as you go, **not from memory at the end of the day**. Use written notes if you forget to take your diary with you and fill out your diary ASAP. Each diary day covers a 24hr period, so please include any food or drinks that you may have had during the night. Remember to include foods and drinks between meals (snacks) including water.

Overleaf you can see an example day that have been filled in. These examples show you how we would like you to record your food and drink, and how to record a homemade dish.

Please document what you ate & drank in as much detail as possible, Remember If it has passed your lips record it! ☺

It only takes a few minutes for each eating occasion!

We thank you for your efforts in filling out this diary.

Recipes / Takeaways

Write in recipes of ingredients of homemade dishes or take-aways

Name of Dish Fairy Cakes	**Serves:** makes 20 cakes
Ingredients – Description, Brand,	**Amounts**
Tate & Lyle caster sugar	175g
Anchor butter, unsalted	175g
ASDA, free range eggs	3 eggs, 172g (no shell)
ASDA Self raising flour	175g
Co-op Baking powder	1 teaspoon
Silver Spoon Icing Sugar	140g
Water	10ml
Description of cooking method Mix all ingredients (1-5) together, then separate into 20 equal portions in cup-cases, cook in oven for 15mins Mix ingredients 6 and 7, pour on top of individual cakes after they are cooked and cooled.	

Name of Dish Big Joe's 16" Meat Feast pizza	**Serves:** 2 equal portions
Ingredients	**Amounts**
Deep pan pizza base	16 inch, weight unknown
Tomato Sauce based	1 ladle
Green peppers	Half green bell pepper
Spicy salami	~ 12 large slices
Pepperoni	~ 20 small slices
Tandoori chicken pieces	~ half chicken breast
Beef meatballs	6 small meatballs
Onion	Half
Garlic mayo dip	75ml pot
Description of cooking method Oven cooked	

Time	Item / Description	Brand / variety	Preparation	Amount	Left-overs
07.00	Coco pops	Asda		82g	
	Skimmed Milk	Asda		322g	
	Pure Orange Juice, not from concentrate	Tropicana – with the bits		1 large Glass - 457g	
09.15	Crunchy Granola Oats and Honey flavour Cereal Bar	Nature Valley		1 bar – 42g	
	Mug of strong tea	Tetley	1 bag Water Sugar Semi milk	 226g 5g 27g	
10.30	Apple	Golden Delicious	Raw	120g	17g core
12.00	Sandwich	Home made			
	Bread (Tesco the finest)	Tesco, sliced, multigrain		2 slices – 98g total	
	Tomato	On the vine		84g	
	Lettuce	Iceberg		35g	
	Chicken Sandwich		Previously roasted leftover chicken breast meat, no skin	105g	
	Butter	Anchor		8g - Thin layer	
	Cucumber	Market		47g	
	Mug of strong tea – Same as above	Same as above			
14.30	Can of Coca-Cola zero	Coca-Cola		1 can 330ml	
15.45	Grande Latte	Starbucks	Extra shot of coffee + vanilla syrup	Grande size	
15:45	Chocolate Brownie	Starbucks		154g	
16.30	Water	Tap		1 pint	
18.30	Pizza - meat feast	Big Joe's – see recipe	Takeaway	Half of 16 inch	
18.45	Diet Lemonade	Aldi		1.5pints	
20.00	Beer normal strength	Carlsberg 5%		4 pints	
22.00	Red-bull, normal	(not diet)		300ml	
	Vodka	Smirnoff		Double measure	
22.30	Toast (Tesco the finest)	Tesco, sliced, multigrain	toast	2 slice	
	Baked beans.	Tesco	Microwave	420g	
Notes	This day I drank more alcohol than normal as it was a colleagues birthday				

Time	Item / Description	Brand / variety	Preparation	Amount	Left-overs

Write in recipes of ingredients of homemade dishes or take-aways

Name of Dish	**Serves:**
Ingredients – Description, Brand,	**Amounts**
Description of cooking method	

Name of Dish	**Serves:**
Ingredients	**Amounts**
Description of cooking method	

7.3 24 hr Physical Activity Diary

We would like to find out about your normal physical activity levels.

Please fill in this diary continuously throughout the day, starting from when you get out of bed in the morning to when you go back to bed at night. The diary is split into the hours of the day, and then four fifteen-minute periods for that hour. In each box, write the code number (see below) which corresponds to the activity which you have carried out during this fifteen minute period. Please fill in **all** the boxes.

Make any notes you feel appropriate during the hours or at the bottom.

The first page is an example which has been done for you.

Please do not wait until the end of the day to fill this out – fill out as you go!

Code Number	**Description and Activity examples**
1. Sleeping	Sleeping, resting in bed, or lying down.
2. Lying down (not sleeping)	Resting in bed, lying down (still awake)
3. Sitting	Sitting, eating, watching TV, reading, listening to music, writing, sewing, talking on phone etc.
4. Standing or Driving	Standing fairly still for example in a Que, or showering, driving a car
5. Light activity / standing with light activities	Standing tasks – dish washing, cooking, vacuuming, ironing, playing a musical instrument, dressing, yoga, slow walk <2.5mph
6. Light movements / light manual work	Walking at a moderate pace (2.8 – 3.5mph), playing with children (moving), brushing/cleaning, gardening, washing windows.
7. Leisure & sports in a recreational environment **low** intensity	Golf, bowling, darts, very gentle cycling, juggling, non-competitive volleyball, table tennis, tai chi, archery, yoga
8. Manual work at a moderate intensity	Digging, sawing, mowing lawn, walking 3mph whilst carrying 10kg, cycling ~10mph
9. Leisure & sports in a recreational environment **low to moderate** intensity	Cycling >10mph, Heavy Resistance (weight) training, hard exercise class, ballet, basketball, volleyball, wrestling, Tennis (doubles), general dancing
10. Leisure & sports in a recreational environment **moderate** intensity	Hockey, fencing, Cricket, Badminton (competitive), Squash, Tennis (singles) Gym Cross-trainer, rock climbing, circuits class, intense aerobics, rowing, recreational football, lacrosse, running 5mph
11. Leisure & recreational sports at a **high** intensity (competitive)	Swimming fast laps, hard hiking, water polo, boxing, cycling > 14mph, canoeing, football, Rugby etc., running 7mph
12. Intense manual work, **very high** intensity sports:	Running > 9 mph, cycling >19 mph, competitive rowing, boxing

Date __/__/__						For Office Use		
Time	0 - 15 mins	16 - 30 mins	31 - 45 mins	46 - 60 mins	Activity / Intensity notes	Code	MET	EE
00:00-01.00	1	1	1	1				
01:00-02:00	1	1	1	1				
02:00-03:00	1	1	1	1				
03:00-04:00	1	1	1	1				
04:00-0500	1	1	1	1				
05:00-06:00	1	1	1	1				
06:00-0700	1	1	1	1				
07:00-08:00	1	1	1	1				
08:00-09:00	1	1	1	2				
09:00-10:00	2	4	4	3				
10:00-11:00	3	3	3	4				
11:00-12:00	3	3	3	3	Working from home and listening to radio			
12:00-13:00	3	3	3	3				
13:00-14:00	5	5	3	3	Walk to lectures			
14:00-15:00	4	3	4	3				
15:00-16:00	3	3	3	3	Lectures			
16:00-17:00	5	3	2	2				
17:00-18:00	5	3	3	3	Reading course work			
18:00-19:00	3	3	3	4				
19:00-20:00	5	5	3	4	Deep House clean!			
20:00-21:00	10	10	10	10	Hockey training			
21:00-22:00	4	4	3	5				
22:00-23:00	3	2	2	1				
23:00-24:00	1	1	1	1				

Date __/__/__						For Office Use		
Time	0 - 15 mins	16 - 30 mins	31 - 45 mins	46 - 60 mins	Activity / Intensity Notes	Code	MET	EE
00:00-01.00								
01:00-02:00								
02:00-03:00								
03:00-04:00								
04:00-0500								
05:00-06:00								
06:00-0700								
07:00-08:00								
08:00-09:00								
09:00-10:00								
10:00-11:00								
11:00-12:00								
12:00-13:00								
13:00-14:00								
14:00-15:00								
15:00-16:00								
16:00-17:00								
17:00-18:00								
18:00-19:00								
19:00-20:00								
20:00-21:00								
21:00-22:00								
22:00-23:00								
23:00-24:00								

7.4 Questionnaire to determine menstrual cycle phase

Taken from the visit 2 data collection sheet from study 1.

1.Have you had regular periods in the last 2 years?Yes □No □

2.Typically how long is your menstrual cycle, from day 1 of menses/period to day one of the next period? _____Days

3.Is the above time the same between periods? Yes □No □

4.If the above was No, please state the irregularity: _____

_____

5.How many days does your menstrual (blood) flow last? _____ Days

6.Number of days since the start of your last period? _____Days

7.5 General health questionnaire

The University of Birmingham

School of Sport and Exercise Sciences

General Health and Screening Questionnaire

Phone/Email:.....

Name of the responsible investigator for the study:

.....

Please answer the following questions. If you have any doubts or difficulty with the questions, please ask the investigator for guidance. Your answers will be kept strictly confidential.

1.	You are.....	Male	Female
2.	What is your exact date of birth? Day..... Month.....Year 19..... So your age is..... Years		
3.	When did you last see your doctor? In the:		

	Last week..... Last month..... Last six months..... Year..... More than a year.....		
4.	Are you currently taking any medication?	YES	NO
5.	Has your doctor ever advised you not to take vigorous exercise?	YES	NO
6.	Has your doctor ever said you have “heart trouble”?	YES	NO
7.	Has your doctor ever said you have high blood pressure?	YES	NO
8.	Have you ever taken medication for blood pressure or your heart?	YES	NO
9.	Do you feel pain in your chest when you undertake physical activity?	YES	NO
10.	In the last month have you had pains in your chest when not doing any physical activity?	YES	NO

11.	Has your doctor (or anyone else) said that you have a raised blood cholesterol?	YES	NO
12.	Have you had a cold or feverish illness in the last month?	YES	NO
13.	Do you ever lose balance because of dizziness, or do you ever lose consciousness?	YES	NO

14.	a) Do you suffer from back pain b) if so, does it ever prevent you from exercising?	YES YES	NO NO
15.	Do you suffer from asthma?	YES	NO
16.	Do you have any joint or bone problems which may be made worse by exercise?	YES	NO
17.	Has your doctor ever said you have diabetes?	YES	NO
18.	Have you ever had viral hepatitis?	YES	NO
19.	Do you know of any reason, not mentioned above, why you should not exercise?	YES	NO
20.	Are you accustomed to vigorous exercise (an hour or so a week)?	YES	NO
21.	Do you take part in physical activity one or more times a week?	YES	NO

I have completed the questionnaire to the best of my knowledge and any questions I had have been answered to my full satisfaction.

Signed:..... **Date:** .....

7.6 Detailed metabolomics methods

The following section was written and produced by Dr Giovanny Bianco and Dr Warwick Dunn describing the methods used at the Phenome Centre Birmingham for processing and analysis of the metabolomics data presented in Chapter 5.

Collection and preparation of specimens

Plasma samples were collected by GF and transferred to the Phenome Centre Birmingham on dry ice. Biological characteristics of the subjects enrolled in this study are described in **Table 5-1 Participant characteristics**.

Chemicals

Acetonitrile (ACN), Methanol (MeOH), Isopropanol (IPA) and HPLC-MS quality water (H_2O) were obtained from Fisher Scientific (UK). Ammonium acetate, ammonium formate, LC-MS quality formic acid, and low binding tubes were obtained from Sigma Aldrich (UK).

Metabolite extraction

Metabolites from plasma samples were extracted applying a monophasic extraction protocol in a randomised order to produce 90 extractions. For each sample, 50 µL of plasma were transferred to an Eppendorf tube (Eppendorf, Cambridge, U.K.). Into each tube at room temperature, the extraction solvent was added in a ratio of 4:1 to precipitate the proteins. 100% acetonitrile and 100% isopropanol were used as extraction solvents for polar and lipid

metabolites, respectively. Samples were centrifuged at 14,000 x *g* for 15 minutes at 4°C and 150 µL of the supernatants were then transferred into separate glass HPLC vials for UHPLC-MS analysis. Remaining aliquots of the extraction solutions were collected in to a single pooled QC sample of which 200 µL was transferred to a new HPLC glass vial and analysed as described for the biological samples.

Untargeted metabolomics analysis of plasma

Ultra High Performance Liquid Chromatography-Mass Spectrometry (UHPLC-MS) analysis was performed applying an Ultimate3000 RSLC UHPLC system coupled to an electrospray Q-Exactive Focus mass spectrometer operating in positive and negative ion modes. MS parameters were adjusted as follows: Resolution 70,000 (FWHM at m/z 200), AGC target 1 x 10^6, Scan Range (m/z) 75–1050, Sheath gas 50, Auxiliary gas 20 and Capillary temperature 275 °C. For positive mode ionisation: Source voltage 4.5 kV, Capillary voltage 40 V, Tube lens voltage 70 V and Skimmer voltage 20 V. For negative mode ionisation: Source voltage -3.5 kV, Capillary voltage -40 V, Tube lens voltage -70 V, Skimmer voltage -20 V. Mass calibration was performed for each polarity immediately before each analysis batch.

For chromatographic separation, 2 µL of each extracted sample were injected on to the UHPLC system. Two different columns were used. For polar compounds, an Accucore-150-Amide-HILIC (100 x 2.1 x 2.6 µm, Thermo Scientific) was used. Mobile phase A and B for the HILIC method consisted in 10 mM ammonium formate in 95% ACN and 10 mM ammonium formate in 50% ACN, respectively. Metabolites were separated using a multi-step gradient

elution program starting in 99% A up to 5% of B in 8 min, where the composition was maintained for 2 min, a final step where the column was re-equilibrated to reach the initial back pressure was then performed. Total analysis time was 15 min, column temperature was 30°C and the flow rate was 500µL min^{-1}. Separation of lipids species was performed using a Hypersil Gold C_{18} column (100 x 2.1 x 1.9 µm, Thermo Scientific). Mobile Phase A consisted in a 10 mM solution of ammonium acetate in H_2O/ACN (60:40), and mobile phase B was a solution containing 10mM ammonium acetate in IPA/ACN (90:10). The multi-step gradient separation started at 60% A up to 99% in 8 min, where the system was maintained for 2 min. Subsequently, the composition of A was set at 60% to re-equilibrate the column for 5 min. The column was maintained at 55°C and the flow rate was 250µL min^{-1}. All samples were analysed in a random order in one analytical batch with 10 QC samples analysed at the start of the analytical batch, after every 6 biological samples and with 2 QC samples analysed at the end of the analytical batch. Two blank samples were also analysed.

Data pre-processing and analysis

UHPLC-MS raw data files (.RAW) were converted to a .mzML format by using the MS-Convert software available in the ProteoWizard package (available at http://proteowizard.sourceforge.net/tools.shtml). Data deconvolution was then performed using XCMS to provide a 2D matrix of chromatographic peaks responses where each peak was defined by the *m/z* ratio and retention time (Dunn et al. 2008). This 2D matrix was exported as a .csv file for data analysis. Metabolites were annotated to levels 2 of the MSI

reporting standards (Sumner et al. 2007) applying PUTMEDID_LCMS (Brown et al. 2011). Data filtering was performed to remove all metabolite features with more than 40% missing values for all QC samples. Considering the relative standard deviation for QC samples analysed from injection 9 onwards, features were also removed when the calculated relative standard deviation was greater than 20% (Dunn, Broadhurst, Begley, et al. 2011). The acquired LC-MS spectra were manually filtered in this was to achieve data of high quality prior to statistical analysis. Statistical analysis was performed in the software package MetaboAnalyst (Xia et al. 2012) with no missing value imputation, normalisation to the sum of peak areas for each sample and with no transformations or scaling performed. Where two classes were compared a non-parametric Mann-Whitney U test was performed with correction for false discovery performed applying the Benjamini-Hochberg method. Where three classes were compared a parametric one-way ANOVA test was performed with correction for false discovery performed applying the Benjamini-Hochberg method. Fold changes were calculated applying the mean response for each class.

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