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The influence of the menstrual cycle on exercise performance and stable isotopic tracer measures of fat metabolism

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CHAPTER 1

INTRODUCTION

Outline of the introduction

Part one of the introduction presents a brief description of the menstrual cycle as a system that functions to promote optimal capacity for reproduction in women; with emphasis on the cyclical fluctuation of the ovarian hormones. Part two presents a detailed review of the effects of the ovarian hormones that are not directly associated with reproductive function, but may have relevance for endurance capacity and performance. The main focus is on the influence of the ovarian hormones (as they naturally occur during the various menstrual phases) on substrate metabolism during exercise at submaximal intensities. Other physiological systems (such as the respiratory, thermoregulatory and cardiovascular systems) that may also be modulated by the ovarian hormones and could alter exercise performance are briefly addressed. Previous studies that have compared exercise performance between menstrual phases are reviewed. Part three presents background information on the methodology used in two of the ensuing studies (Chapters 2 and 3) presented in the thesis for assessing free fatty acid oxidation and kinetics during exercise. Finally, in part four the introduction is concluded by a brief summary.

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Part 1

1.1 The menstrual cycle

1.1.1 Overview of menstrual cycle physiology

The ovarian hormones, oestrogen and progesterone, are secreted from the ovaries and to a lesser extent from the adrenal glands in women (Lebrun 1994). 17β-Oestradiol is the primary oestrogen secreted, but may be metabolised further to from estrone and estriol, which are less potent oestrogens (Lebrun 1994). Alternatively, the oestrogens may follow a different metabolising pathway to form the catecholoestrogens, 2- and 4-hydroxyoestrogens that are then methylated to form monomethylesters (the methoxyoestrogens) (De Crĕe et al. 1997).

Secretion of the reproductive hormones follows a repetitive cyclical pattern over the course of, on average, 23 to 38 days (Reilly 2000) in normally menstruating (eumenorrhoeic) women. This circamensal rhythm known as the menstrual cycle is broadly divided into two phases- the follicular phase (FP) and the luteal phase (LP), which are separated by ovulation.

The onset of menstruation designates the first day of the cycle (Figure1). The system involved in the regulation of the cycle is termed the hypothalamic-pituitary-ovarian axis and is thoroughly reviewed by Birch (2000) and Reilly (2000). Briefly, during the follicular phase, the hypothalamus secretes gonadotrophin-releasing factor (GnRH) that stimulates the anterior pituitary gland to secrete follicle stimulating hormone (FSH) and luteinising hormone (LH). FSH stimulates maturation of a follicle in the ovary into a graafian follicle. FSH and LH stimulate the ovary to secrete oestrogen such that circulating oestrogen concentrations gradually increase one week before ovualtion and then dramatically increases in a surge that peaks 24 hours before ovulation. This surge in oestrogen stimulates further release of LH by positive feed-forward stimulation and causes a mid-cycle surge in LH that sets off ovulation. The LH surge commences 36 hours before ovulation occurs (Buffenstein et al. 1995). To a lesser extent, FSH concentration also rises at ovulation. The LP follows ovulation with the conversion of the ruptured follicle into the corpus luteum, which produces large amounts of oestrogen and progesterone. However, the magnitude of increase in oestrogen in the LP is not as

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high as during the short surge that occurs prior to ovulation, but the relatively high oestrogen levels in the LP persists for longer. During the LP the secretion of GnRH, LH and FSH are suppressed by negative feedback of oestrogen and progesterone on the hypothalamus and pituitary gland. Oestrogen and progesterone function to prepare the uterine wall for implantation of a blastocyte and if after roughly seven days implantation has not occurred, the corpus luteum regresses and the ovarian steroid hormone concentrations start to decline. When progesterone levels have dropped sufficiently, menstruation and a new cycle commences. During menstruation approximately two thirds of the endometrial lining is shed. During the late stages of the LP when oestrogen and progesterone levels have reduced substantially, the negative feedback on FSH and LH secretion is lifted and thus the increase in FSH even during the last two days before menstruation will set off the development of a new follicle that will continue into the next cycle (Buffenstein 1995).

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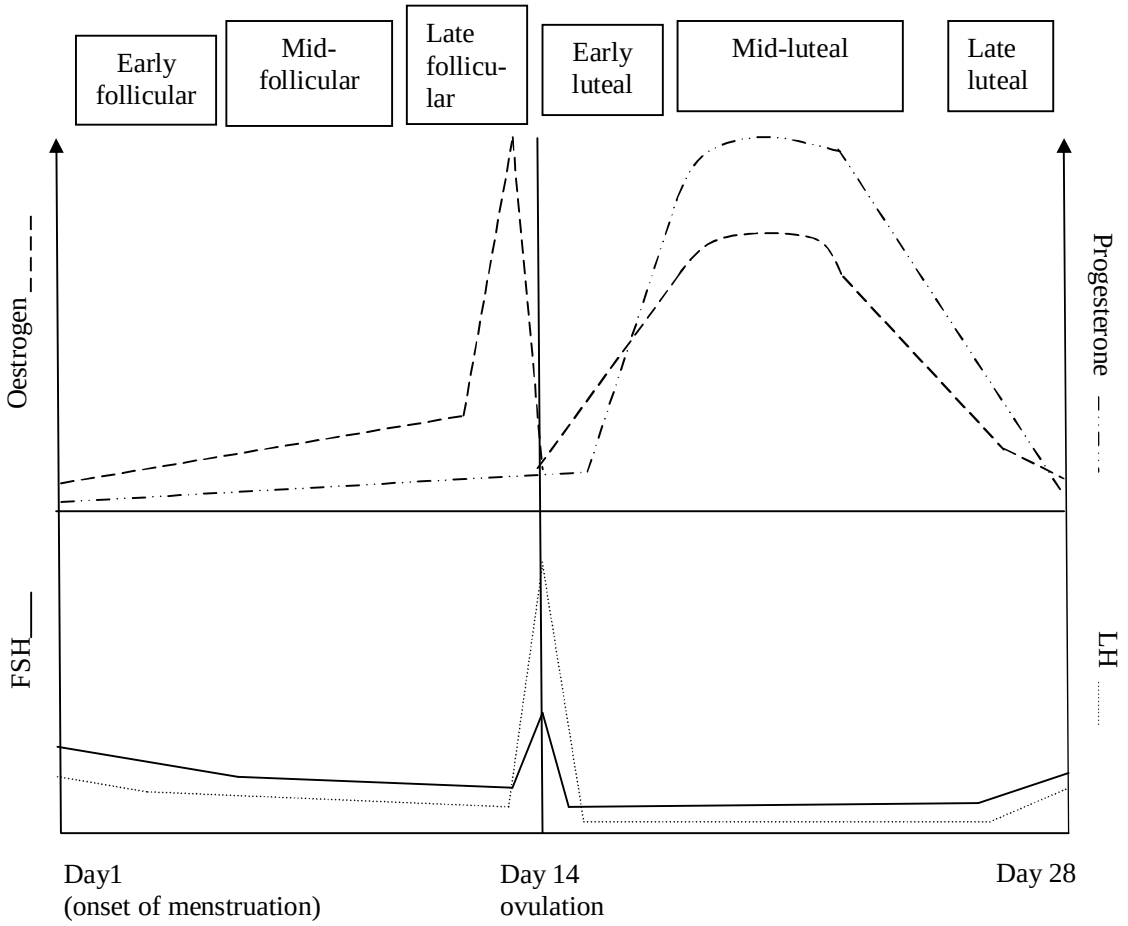

Figure 1. Diagrammatic representation of the cyclical changes in the female sex hormones that characterise the various menstrual phases. FSH- follicle stimulating hormone; LH- luteinising hormone.

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1.1.2 Menstrual phases of a eumenorrhoeic cycle

According to the periodic rise and fall in oestrogen and progesterone the menstrual cycle can be divided into the following phases (Figure 1):

Early follicular (EF)- Roughly the first week of the cycle starting on the first day of menses, characterised by very low oestrogen and progesterone concentrations. Some studies (such as Seebauer et al. (2002)) choose to differentiate between the period during which menses occurs and days post menses and so only refer to the EF phase as the few days succeeding menses. However, as oestrogen and progesterone concentrations remain fairly low during and for the few days after menses, most studies (including the work presented in this thesis) refer to the entire first week of the cycle as EF (for example Lebrun et al. 1995).

Mid-follicular (MF)- Characterised by gradual increase in oestrogen that precedes the pre-ovulatory spike in oestrogen and corresponds to roughly day 8 to 11 in an average 28 day cycle (Dombovy et al. 1987).

Late follicular (LF)- Encompasses the pre-ovulatory surge in oestrogen and the LH surge up to the time of ovulation (day 12 to 14 of an average 28 day cycle). Some studies refer to this period as the peri-ovulatory (Hornum et al. 1997) or even as the ovulatory phase (Hackney et al. 1991).

Early luteal (EL)- The first few days following ovulation (4 days post ovulation) and is characterised by increasing concentrations of oestrogen and progesterone (Seebauer et al. 2002).

Mid-luteal (ML)- Four to nine days following ovulation and includes the highest oestrogen and progesterone concentrations of the LP (Lebrun et al. 1995).

Late luteal (LL)- When oestrogen and progesterone concentrations are decreasing just prior to menses from approximately 11 days following ovulation up to the day before menses (Seebauer et al. 2002).

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The main menstrual phases under investigation in the studies presented herein include: EF, LF and ML phases.

1.1.3 Menstrual cycle characterisation: eumenorrhoea, oligomenorrhoea or amenorrhoea

A eumenorrhoeic cycle is a menstrual cycle that occurs regularly and is capable of promoting the successful implantation of a fertilised ovum in order to initiate its development (Reilly 2000). Such a cycle requires a functional hypothalamic-pituitary-ovarian axis that stimulates an adequate follicular phase, the occurrence of ovulation and a functional corpus luteum (Keizer and Rogol 1990). Disturbances of the hypothalamic-pituitary-ovarian axis results in cycles that can be highly irregular, anovulatory or have inadequate corpus luteum function (Keizer and Rogol 1990). It is even possible for the cycle to be completely suppressed in which case the ovarian steroids remain consistently low and such cases are characterised by the complete absence of menstruation and is termed amenorrhoea. Oligomenorrhoea is the term used for the condition when menstruation occurs infrequently, such as every four months, or unevenly spaced menses. Factors such as regular intensive exercise, calorie restrictions and lifestyle stress may cause a disturbance in the hypothalamic-pituitary-ovarian axis resulting in oligomenorrhoea or amenorrhoea or inadequate luteal phase (Keizer and Rogol 1990). Hormonal contraceptives alter this regulatory system in order to prevent the occurrence of ovulation.

1.1.4 Common indicators used as evidence of a eumenorrhoeic cycle

The following methods are used to test for eumenorrhoea:

Biphasic body temperature profile- The elevated progesterone concentration that characterise the LP induces an increase in body temperature set point, increasing basal body temperature by 0.3-0.5 ^{o}C (Reilly 2000). Daily body temperature readings taken at the same time of day over the course of a cycle should result in a biphasic temperature pattern. Such temperature charts may help to estimate or predict the possible day of ovulation. However, in some women the post ovulatory increase in progesterone does not produce an increase in body temperature set point and thus some ovulatory cycles do not present with the typical biphasic temperature pattern. Therefore this method is

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useful in assisting with estimating the timing of menstrual phases, but cannot be used to verify eumenorrhoea or confirm the occurrence of a menstrual phase during an experimental trial.

LH surge- Commercial kits are available for identifying LH concentration in mid-stream urine samples as typically occurs during the pre-ovulatory LH surge and results are read as a simple positive or negative. A positive result provides evidence of an ovulatory menstrual cycle.

Serum progesterone concentration- A circulating progesterone concentration of more then 10 nmol/L in the ML phase is considered the minimum value required to indicate that ovulation has taken place (Keizer and Rogol 1990).

Serum oestrogen concentration- The various menstrual phases are characterised by a fixed acceptable range for oestrogen concentration. The accepted concentration ranges for the respective menstrual phases (Coat-A-Count, Diagnostic Products, Los Angeles, CA) used in the work presented herein are as follows:

EF- 37-220 pmol/L

LF- 360-1377 pmol/L

ML- 220-955 pmol/L.

Regular cycles and the length of the LP- Cycles should occur regularly over 23 to 38 days (Reilly 2000), although the length of the cycle can vary slightly from one cycle to the next. This variability is largely due to changes in the FP length, as the length of the LP is more consistent (Keizer and Rogol 1990). However, even apparently normal cycles that occur regularly and are identified as ovulatory can have aberrations; such as a short LP or LP insufficiency. A shortened LP is a consequence of aberrant folliculogenesis while LP insufficiency is due to inadequate functioning of the corpus luteum (Keizer and Rogol 1990). The LP must last for 10 days or more and progesterone must increase sufficiently to indicate adequate LP function (Keizer and Rogol 1990).

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Less frequently used methods for confirmation of eumenorrhoea include:

Cervical mucus assessment- Post ovulation increase in progesterone stimulates the development of mucus-secreting glands. Thus an increase in mucus in the cervix is an indication of ovulation (Reilly 2000).

Ultrasonic assessments- The progression of folliculogenesis and the occurrence of ovulation can be assessed.

Only those studies that have confirmed menstrual phase comparisons by measurements of serum progesterone concentration have been included in this literature review. In the studies presented herein, temperature charts and detection of the LH surge were used to predict the days for testing for each menstrual phase, which was subsequently confirmed by resting oestrogen and progesterone concentration.

1.1.5 Influence of exercise on the concentration of the ovarian hormones

Oestrogen and progesterone

The concentration of oestrogen increases as a result of exercise during the EF, MF, LF, and ML phases (Jurkowski et al. 1978; Kanaley et al. 1992; Lavoie et al. 1987). A noticeable increase in progesterone concentration is evident due to exercise in the luteal phase (Jurkowski et al. 1978; Kanaley et al. 1992; Lavoie et al. 1987) and occasionally a modest increase is reported during exercise in the follicular phase (Kanaley et al. 1992). The magnitude of increase in ovarian hormone concentration over resting values may be related to the intensity of exercise and it is presumed to be a result of a decrease in metabolic clearance rate due to reduced hepatic blood flow during exercise (Jurkowski et al. 1978).

LH and FSH

Circulating concentration of these hormones are generally unaffected by exercise (Bonen et al. 1983; Jurkowski et al. 1978). However, Lavoie et al. (1987) reported a significant decrease in LH during the MF and ML phase in response to exercise under conditions of metabolic stress (carbohydrate-depleted and overnight fasted condition).

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Part 2

1.2 Non-reproductive actions of the ovarian steroid hormones

Although oestrogen and progesterone's primary role is maintenance of reproductive function in women, these hormones have an affect on a number of other physiological functions; namely metabolism, respiration, thermoregulation, cardiovascular and even maintenance of cellular membrane integrity. Some of these actions may have relevance for optimising conditions during gestation. However, these "secondary actions" of the ovarian hormones may also have implications for athletic performance and therefore the influence of the ovarian hormones on each of these systems is thoroughly addressed in this review. In particular, a detailed overview of the metabolic manipulation of the three main macronutrients (carbohydrate, fat and protein) under conditions of varying ovarian hormonal milieu is presented. The main emphasis is a comparison between menstrual phases; however, where appropriate, studies employing ovarian hormone supplementation in humans and animals are presented. In addition, some gender comparison studies are included when ovarian hormone supplement or menstrual phase comparison studies are lacking or insufficient.

1.2.1 **Ovarian hormones and metabolism**

Both oestrogen and progesterone concentrations are reported to alter metabolic responses. However, in this respect progesterone displays largely anti-oestrogenic affects (Campbell and Febbraio 2001; Campbell and Febbraio 2002; D'Eon et al. 2002; Hatta et al. 1988). D'Eon et al. (2002) has proposed that a metabolic response to changes in the ovarian hormones occurs only when the ratio of oestrogen to progesterone is sufficiently elevated and the magnitude of the increase in oestrogen from the EF to the phase of comparison such as LF or ML is of the order of approximately 2-fold. Others consider nutritional status to be an overriding variable since most variations in metabolism are reported when subjects participate in a study following an overnight fast, whereas a positive nutritional state has been reported to negate these affects (Campbell et al. 2001).

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1.2.1.1 Ratio of fat to carbohydrate oxidation by indirect calorimetry

Basic concepts of the use of indirect calorimetry to estimate substrate utilisation

The rate of oxygen consumption (VO_2) and carbon dioxide production (VCO_2) can be estimated under steady state condition from measured oxygen and carbon dioxide content of expired air. Based on stoichiometric equations, the relative proportion of VCO_2 to VO_2 represents whole body cellular substrate oxidation. The respiratory exchange ratio (RER; VCO_2/VO_2 estimated from expired air), approximates the respiratory quotient, (RQ; VCO_2/VO_2 estimated at the tissue level) under steady state conditions. As the amount of protein oxidised to yield energy is small in comparison to fat and carbohydrate oxidation, the non protein respiratory exchange ratio is often used where the proportion of fat to carbohydrate is estimated without regard for the contribution of protein oxidation to the resultant ratio. An RER that tends towards 0.707 indicates predominant fat oxidation while an RER closer to 1.0 represents mostly carbohydrate oxidation. The theory supporting the use of RER to estimate whole body substrate utilisation and the various conditions that invalidate the underlying assumptions have been thoroughly reviewed by a number of authors (Ferrannini 1988; Frayn et al. 1983; Jéquier et al. 1987; Simsons and DeFronzo 1990).

Variation in RER between menstrual phases

A number of studies have found RER to vary between menstrual phases. Most report a lower RER in the ML phase compared to the MF phase at rest (Nicklas 1989) and during exercise (Campbell et al. 2001; Dombovy et al. 1987; Zderic et al. 2001) or during exercise in the LF phase compared to the MF phase (Hackney 1991). These findings would suggest that either oestrogen alone (as in the LF phase) or oestrogen and progesterone when present together as in the ML phase, promote fat utilisation or suppress carbohydrate oxidation. In contrast, a number of other studies have reported no significant changes to the RER between menstrual phases either at rest or during exercise, or both (Bailey et al. 2000; De Souza et al. 1990; Hessemer and Brűck 1985, Horton et al. 2002; Kanaley et al. 1992; Suh et al. 2002). Administering exogenous oestrogen to amenorrhoeic women (Ruby et al. 1997) or men (Carter et al. 2001) produced no significant difference to the RER at rest or during exercise compared to

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placebo treatment. In a recent study the ovarian steroid hormones were controlled pharmacologically in eumenorrhoeic women to induce conditions of low sex hormones versus high oestrogen versus high oestrogen and progesterone, so as to mimic ideal conditions of the various menstrual phases (D'Eon et al. 2002). In this study the oestrogen alone condition resulted in significant reductions to exercising RER while the oestrogen and progesterone combination condition presented similar RER values to the baseline or low hormone condition.

Therefore, the current literature on the influence of the ovarian steroid hormones on whole body substrate utilisation appears to be inconsistent. However, based on observations of studies such as that of Campbell et al (2001) many researchers suggest that nutritional status is an overriding variable influencing substrate utilisation. For example Campbell et al (2001) showed that the reduction in exercising RER in the luteal phase following an overnight fast was reversed when a carbohydrate supplement was ingested during exercise. In support of this theory, Zderic et al. 2001 observed variations in RER between phases when measurements were performed following an overnight fast. However, some researchers who have found differences in RER between menstrual phases (Dombovy et al. 1987) or following exogenous ovarian hormone treatment (D'Eon et al 2002), studied subjects who were only 3 to 4 hours post-absorptive (non-fasted). Others have found no difference in RER despite subjects fasting overnight (Carter et al. 2001; Horton et al. 2002; Kanaley et al. 1992). Therefore, there may be more to the controversy of inconsistent reports of RER between menstrual phases than simply differences in the nutritional status of participants. D'Eon et al. (2002) suggest that magnitude of increase in oestrogen from one phase to another and the relative increase in oestrogen over progesterone in the luteal phase are important variables to consider. Such a theory could explain why D'Eon et al. (2002) observed a lower RER during exercise only with the oestrogen-only treatment and not with the combined oestrogen and progesterone treatment, as the exogenous dose of progesterone administered resulted in supra-physiological levels of progesterone that reduced the oestrogen to progesterone ratio (E/P).

The RER measurement presents only a crude estimation of whole body substrate utilisation and can be altered by factors other than just carbohydrate, fat and protein

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oxidation. For example, triacylgycerol synthesis increases RER. That is, the first step of the incorporation of fatty acids into triacylglycerol molecules involves the condensation of the fatty acid into fatty acyl CoA (catalysed by the enzyme fatty acyl CoA synthetase) which results in the liberation of carbon dioxide and water (Van der Vusse and Reneman 1996) and consequently increases RER. Interestingly, animal studies have found that oestradiol administered to male rats elevated intramuscular triacylglycerol synthesis during exercise (Ellis et al. 1994). Furthermore, under fasted conditions there is an increased flux through gluconeogenic and ketogenic pathways that will reduce the RER (Jéquier et al.1987). Non metabolic carbon dioxide production that occurs during intense exercise for the purpose of the maintenance of acid-base status will elevate RER levels and can therefore cause a false over-estimation of carbohydrate oxidation (Jéquier et al. 1987).

1.2.1.2 Influence of menstrual phase on the hormonal regulators of metabolism

(Refer to Table 1 for a concluding summary.)

1.2.1.2.1 Insulin

Insulin is a peptide hormone secreted by beta cells of pancreatic islets of Langerhans. Its metabolic effects include stimulation of glycogenesis, lipogenesis or free fatty acid (FFA) storage and protein synthesis by promoting cellular uptake of the respective substrates (Vander et al. 1996). Insulin inhibits gluconeogensis and lipolysis. Insulin secretion is primarily regulated by blood glucose concentration. During exercise, however, insulin secretion is blunted due to increases in epinephrine and sympathetic nerve activity, thereby reducing insulin's inhibition of lipolysis (Jeukendrup et al. 1998).

Oestrogen has a direct trophic effect on the pancreatic beta islets and can protect against diabetes mellitus in rats (Godsland 1996). A recent study (Latour et al. 2001), reported lower basal insulin concentration in ovarectomised rats with oestrogen treatment than with placebo treatment, suggesting that oestrogen improves insulin sensitivity. However, they found no change to glucose tolerance or insulin response when a large glucose dose was administered (Latour et al. 2001). These findings concur with that of a

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previous study (Hansen et al. 1996), in which control female rats compared with ovarectomised (i.e. ovarian hormone deficient) females tended to have greater glucose uptake when exposed to submaximal levels of insulin indicating greater insulin sensitivity. However, maximum insulin-stimulated glucose transport activity was not altered by ovarectomy and hence insulin responsiveness is not influenced by the presence of ovarian hormones (Hansen et al. 1996) thus agreeing with the results of the glucose tolerance test of the former study (Latour et al. 2001). The non-significant trend towards improved insulin sensitivity in the rats with intact ovaries (Hansen et al. 1996) is most likely attributable to an oestrogen-action, as progesterone is believed to cause insulin resistance (Kalkhoff 1982). While progesterone also causes hypertrophy of the beta islets, this occurs as a consequence of progesterone-induced insulin resistance (Kalkhoff 1982) caused by progesterone reducing glucose transport capacity in various tissues (Campbell and Febbraio 2002). However, the changes in the ovarian hormones between menstrual phases mostly cause no noticeable difference in insulin concentrations at rest and during exercise (Bailey et al. 2000; Suh et al. 2002; Zderic et al. 2001) or with pharmacological treatment of oestrogen (Carter et al. 2001; Ruby et al. 1997).

Some studies have reported differences in insulin concentration between menstrual phases. Particularly, an increase in the LP compared to FP is accompanied by an increase in blood glucose concentration (Bonen et al. 1983; Horton et al. 2002); possibly a result of a low E/P and thus resulting in an overriding progesterone-induced insulin resistance. Oestrogen depletion has also been associated with insulin resistance (Latour et al. 2001) and may explain the higher insulin concentrations reported by D'Eon et al. (2002) in women when the ovarian hormones were pharmacologically suppressed compared to that following oestrogen or oestrogen and progesterone treatments.

1.2.1.2.2 Glucagon

Glucagon is a peptide hormone secreted by alpha cells of pancreatic islets of Langerhans and its role is to increase plasma glucose concentration (Vander et al. 1994). Glucagon acts on hepatic receptors to increase glycogenolysis, gluconeogenesis

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and ketogenesis. The major cue to glucagon secretion is hypoglycaemia, but the sympathetic nervous system and increases in epinephrine concentration also result in an increased glucagon secretion (Vander et al. 1996). Only one study has reported a significant difference in glucagon concentration between menstrual phases. In that study, concentrations were significantly higher in the ML phase compared to the EF phase in fasted subjects at rest and during exercise (Campbell et al. 2001). The causality of this observation is obscure. A further study reported a non-significant trend for glucagon to be lower in the ML phase compared to the MF phase during exercise in a carbohydrate-depleted state and may be attributed to the trend for a lower epinephrine concentration also observed in the ML phase of this study (Lavoie et al. 1987). Others have found no change in glucagon between menstrual phases (Horton et al. 2002; Suh et al. 2002).

1.2.1.2.3 Growth Hormone

Growth hormone is a peptide hormone secreted by the anterior pituitary and has the primary function of enhancing body growth by stimulating protein synthesis (Vander et al. 1994). However, growth hormone has other metabolic affects; that is, it heightens adipocyte sensitivity to lipolytic stimuli, enhances hepatic gluconeogensis and reduces insulin-induced glucose uptake (Vander et al. 1996). The net result of which should be an increased plasma concentration of glucose and free fatty acids. Growth hormone secretion is regulated by the hypothalamic hormones somatostatin, which inhibits growth hormone secretion and growth hormone releasing hormone (which stimulates growth hormone secretion). Various physiological states (such as exercise, stress, fasting, a low plasma glucose concentration and sleep) can stimulate growth hormone secretion by decreasing the secretion of somatostatin and/or increasing that of growth hormone releasing hormone (Vander et al. 1996).

Although studies have reported no significant difference in growth hormone concentration at rest or during moderate intensity exercise between menstrual phases (Kanaley et al. 1992), some have identified a higher growth hormone concentration in the ML compared to the EF phase at rest, during exercise, and at exhaustion (Bonen et al 1983; Nicklas et al. 1989; Zderic et al. 2001). Another report of greater resting and

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exercise GH levels in the peri-ovulatory phase (or LF phase) compared to EF phase provides evidence for the menstrual cycle fluctuations in resting GH and the GH response to exercise, to be related to a change in oestrogen concentration (Hornum et al. 1997). Indeed, this corresponds with the observations of an earlier study by Faria et al. (1992) that monitored resting 24-h serum GH secretion during the EF, LF and ML phase and discovered that the amplitude of the GH pulse secretion and integrated GH concentration was significantly greater in the LF phase than the EF phase, while the ML phase secretion was similar to the EF phase (although also not significantly different from the LF phase). Furthermore, oestrogen concentration correlated positively, while progesterone concentration correlated negatively with GH maximum peak height, thus indicating oestrogen's stimulatory action and progesterone's inhibitory action on GH secretion. The suggested mechanism is via either stimulation of GHRH synthesis or secretion, or heightened sensitivity of somatotrophs to GHRH (Faria et al. 1992). The possibility of ovarian hormones acting by modulating somatostatin secretion is reduced because a greater frequency of pulse secretions would be expected if somatostatin's inhibition of GH secretion were reduced. Instead Faria et al. (1992) observed only greater amplitude of the GH pulse secretion with high oestrogen concentration, while pulse frequency of GH release was similar between menstrual phases.

1.2.1.2.4 Cortisol

Cortisol is a steroid hormone secreted from the adrenal cortex and is the major glucocorticoid responsible for regulating metabolism during fasting. It is secreted in larger amounts during stress; causing increased protein catabolism, increased gluconeogenesis, increased lipolysis and reduced cellular glucose uptake (Vander et al. 1996). Most studies have found no difference in cortisol at rest or during exercise between menstrual phases (Bailey et al. 2000; Galliven et al. 1997; Horton et al. 2002) or with exogenous oestrogen treatment (Carter et al. 2001). Lavoie et al. (1987), however, found cortisol levels to be greater after prolonged moderate exercise in the ML phase compared to the MF phase in overnight fasted, carbohydrate depleted subjects.

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Table 1. Summary of the potential effects of oestrogen and progesterone on the hormonal metabolic regulators

Metabolic hormone	Oestrogen	Progesterone
Insulin	↑ Insulin sensitivity → ↓ [Insulin]	↑ Insulin resistance → ↑ [Insulin]
Glucagon	↓ Glucagon with oestrogen-induced ↓ Epinephrine	?
Growth hormone (GH)	↑ amplitude but not frequency of pulsitile GH secretion→ ↑ [GH]	Possibly ↓ pulse amplitude of GH secretion → ↓ [GH]
Cortisol	Mostly has no effect but under extreme metabolic stress may → ↑ [cortisol]	
Epinephrine	↑ beta adrenergic sensitivity → ↓ [epinephrine]	No effect
Norepinephrine	No effect	No effect

Square brackets [ ] denotes concentration.

1.2.1.2.5 Catecholamines secreted as hormones

Epinephrine

Circulating levels of epinephrine reflect the quantity of this catecholamine that is secreted from the adrenal medalla under various stressors (Vander et al. 1996). The metabolic affect of epinephrine includes increasing circulating glucose, FFA and glycerol concentrations by stimulating glycogenolysis, gluconeogenesis and lipolysis; and thereby acts in opposition to insulin (Vander et al. 1996). Naturally, elevated epinephrine concentration has other physiological actions such as elevating heart rate, metabolic rate, respiratory rate, etc (Vander et al. 1996). However, most studies have found no difference in epinephrine concentration at rest or in response to exercise between menstrual phases (Bailey et al. 2000; Horton et al. 2002; Dean et al. 2003) or between various ovarian hormone treatments (Carter et al. 2001; D'Eon et al. 2002). Ruby et al. (1997) observed lower epinephrine levels at exhaustion from a prolonged

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exercise protocol in overnight fasted amenorrhoeic women following oestrogen treatment compared to the placebo condition. In addition, Lavoie et al. (1987), found a trend for lower epinephrine concentration in the luteal phase compared to the follicular phase at the end of prolonged submaximal exercise in subjects who were carbohydrate depleted and overnight fasted. It may be that oestrogen heightens beta adrenergic receptor sensitivity (Benoit et al. 1982) and thus a similar metabolic response is produced at lower epinephrine levels.

Norepinephrine

The catecholamine, norepinephrine is secreted in small quantities from the adrenal medulla and has similar actions to that of epinephrine. All studies measuring norepinephrine between menstrual phases (Bailey et al. 2000; Dean et al. 2003; Horton et al. 2002; Lavoie et al. 1987) or with ovarian hormone treatment (Carter et al. 2001; D'Eon et al.2002; Ruby et al. 1997) have found no difference in the resting or exercising concentrations.

1.2.1.3 Resultant effect of ovarian hormones on various aspects of carbohydrate metabolism

(Refer to Figure 2 for a flow diagram summary)

1.2.1.3.1 Plasma glucose concentration

A large majority of studies report no significant difference in glucose concentration at rest or during exercise between menstrual phases (Bailey et al. 2000; Bonen et al. 1983; Kanaley et al. 1992; Hackney et al. 1991; Nicklas et al. 1987; Suh et al. 2002) or between pharmacologically elevated oestrogen or oestrogen and progesterone conditions compared to a placebo condition (Carter et al. 2001; D'Eon et al. 2002; Ruby et al. 1997). Blood glucose concentrations have been reported to be elevated at rest and during submaximal exercise (Horton et al. 2002; Zderic et al. 2001) or post exercise (Galliven et al. 1997) in the ML phase compared to the follicular phase (EF or MF and LF phase). This could be related to altered rate of endogenous glucose production or

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rate of glucose uptake or could suggest insulin resistance, or both. Progesterone induces insulin resistance by decreasing GLUT 4 protein content in insulin–sensitive tissue (Campbell and Febbraio 2002) and this may explain the increased glucose concentration at rest. In fact previous studies have found glucose tolerance to be impaired in the luteal phase as a result of progesterone-induced insulin resistance (Elkind et al. 1993; Ezenwaka et al. 1993). However, progesterone also inhibits contraction-stimulated glucose uptake (Campbell and Febbraio 2002) and as this is the major glucose uptake mechanism during exercise, may explain the observed increase in circulating glucose during exercise in the ML phase noted in some studies. Conversely, Campbell et al. (2001) reported exercising blood glucose concentrations to be higher in the follicular phase compared to the luteal phase, albeit in subjects receiving carbohydrate supplement during exercise. This finding is difficult to explain, as isotopic measures of blood glucose kinetics were similar between the luteal and follicular phase in these subjects (Campbell et al. 2001). A further study performed in carbohydrate depleted women found that while blood glucose was maintained during submaximal exercise in the MF phase, concentrations dropped progressively during exercise in the ML phase (Lavoie et al 1987). This observation, however, demonstrates the previously documented capacity for oestrogen and progesterone induced inhibition of gluconeogensis (Matute and Kalkhoff 1973), which would be the prime source of endogenous glucose production during times of carbohydrate depletion without exogenous supplementation.

1.2.1.3.2 Plasma lactate concentration

Some studies have reported a higher blood lactate concentration during or post exercise in the early to mid-follicular phase compared to the mid-luteal phase (Lavoie et al. 1987; Jurkowski et al. 1981; McCracken et al. 1994; Zderic et al. 2001), thus possibly suggesting a greater reliance on glycolysis/glycogenolysis to meet the energy demand during exercise. However, the majority have found no difference in resting or exercising blood lactate concentrations between menstrual phases (Bemben et al. 1995; Bonen et al. 1983, 1991; Campbell et al. 2001; Dean et al. 2003; De Souza et al.; Galliven et al. 1997; Hessemer et al. 1985; Horton et al. 2002; Kanaley et al. 1992; Nicklas et al. 1989;

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Suh et al. 2002) or with pharmacological treatments of the ovarian hormones (Carter et al. 2001; D'Eon et al. 2002; Ruby et al. 1997).

1.2.1.3.3 Muscle glycogen content quantified from muscle biopsies

Estimation of muscle glycogen content by muscle biopsy suggests that the hormone milieu in the luteal phase promotes muscle glycogen storage (Hackney 1990, Nicklas et al. 1989) and reduces muscle glycogen use during exercise (Hackney 1999) with oestrogen being the ovarian hormone implicated for these changes (Hackney 1999; Kendricks et al. 1987). That is, the findings of Nicklas et al. (1989) support the theory of the ovarian hormone promoting glycogen synthesis as they reported greater muscle glycogen repletion following a period of induced glycogen depletion in the luteal phase compared to the follicular phase. Hackney (1990) observed greater resting muscle glycogen concentrations in the luteal phase compared to the follicular phase and observed lower rates of glycogen use in the luteal phase compared to the follicular phase based on biopsies sampled before and after 60 min of exercise at 70%VO_2max (Hackney 1999). In addition, this latter study found that muscle glycogen use during exercise was negatively correlated to oestrogen concentration. Animal studies using oophorectomised rats on oestrogen treatment confirm these findings of oestrogen-stimulated glycogen sparing during exercise (Kendricks et al. 1987).

1.2.1.3.4 Animal studies investigating the effect of the ovarian hormones on carbohydrate metabolism

With the use of radioisotopes, Matute and Kalkhoff (1973) investigated the influence of the ovarian hormones on hepatic carbohydrate metabolism and concluded that both ovarian hormones promote the effects of insulin by increasing insulin concentration to stimulate glycogenesis and suppress gluconeogenesis. They propose that the purpose of such an action on the part of the ovarian hormones is to guarantee the availability of glucose for preferential use by the foetus (in the event of successful conception) when food is withheld while providing alternative substrate such as FFA for maternal consumption. However, progesterone inhibits glucose uptake by reducing GLUT 4 transporter protein content in insulin sensitive tissue and inhibits contraction stimulated glucose uptake (Campbell and Febbraio 2002). While oestrogen does not significantly

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increase GLUT 4 transporter content relative to a condition of ovarian hormone deficiency (Campbell and Febbraio 2002), it may improve insulin sensitivity as previously discussed, by a tendency to increase glucose uptake at submaximal insulin levels (Hansen et al. 1996). Oestrogen is also effective in promoting contraction-stimulated glucose uptake (50% increases are reported with oestrogen replacement relative to oestrogen deficiency by ovarectomy) (Campbell and Febbraio 2002; Hansen et al. 1996). Progesterone, however, is capable of antagonising these positive actions of oestrogen but can be overcome by a sufficiently high oestrogen to progesterone ratio (Campbell and Febbraio 2002). In addition to a larger pre-exercise liver glycogen store with oestrogen treatment when compared to untreated and progesterone-treated ovarectomised rats, oestrogen also promoted greater hepatic glycogenolysis during exercise in response to the greater peripheral demand (noted by the increase in glucose uptake) (Campbell and Febbraio 2002).

Conversely, others have found that oestrogen treatment in male and oophoretomised female rats did not change resting glycogen content in liver, myocardium or red and white vastus and soleus skeletal muscle (Kendricks et al. 1987; Rooney et al. 1993). The different findings with regard to oestrogen's effect on glycogen stores may be a consequence of varying control of diet. In the latter studies oestrogen treatment did however, delay glycogen utilisation during submaximal exercise in some tissues and resulted in major glycogen sparing in all tissues (Kendricks et al. 1987; Rooney et al. 1993). In fact the rats on oestrogen replacement managed 100 min longer time to exhaustion than placebo controls, where exhaustion was characterised by liver glycogen depletion in both groups (Kendricks et al. 1987). Liver glycogen depletion impairs endogenous glucose production and without exogenous glucose supplements will ultimately causes a drop in blood glucose concentration and thus marks the point of exhaustion (Bosch et al. 1993). Hence liver glycogen sparing with oestrogen treatment prolonged endurance capacity by maintaining euglycemia. However, glycogen sparing promoted by elevated oestrogen may be secondary to the oestrogen-mediated increase in the availability of FFA for muscle during exercise (Kendricks et al. 1987; Rooney et al. 1993). Briefly, oestrogen treatment promotes the redistribution of FFA from retroperintoneal fat pads to skeletal muscle causing significant increases in muscle

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triacylglycerol content and plasma FFA concentration compared to placebo treated rats (Rooney et al. 1993).

1.2.1.3.5 Glucose kinetics

In a fasted state, glucose rate of appearance (Ra) is solely determined by endogenous glucose production, which is predominantly controlled by hepatic gluconeogenesis and glycogenolysis. Glucose rate of disappearance (Rd) is dependent on insulin-mediated glucose uptake and contraction-mediated glucose transport, with the latter predominating during exercise. Glucose Ra and Rd are naturally related to each other and primarily influenced by the rate of glucose utilisation (Horton et al. 2002).

A number of studies have found that the Ra and Rd of glucose during exercise is attenuated by either therapeutic increases in circulating oestrogen (Carter et al. 2001; D'Eon et al. 2002; Ruby et al. 1997) or with the coincident rise in oestrogen and progesterone during the ML phase of the menstrual cycle compared to the EF phase (Campbell et al. 2001; Zderic et al. 2001). Therefore, the ovarian hormone-induced decrease in glucose kinetics is most likely an oestrogen-associated effect and is one that progesterone does not antagonise but may in fact potentiate (D'Eon et al. 2002). Glucose Ra was dependant on hepatic glucose production in all of the above studies as the subjects did not receive any form of exogenous glucose during exercise and subjects in all of the above studies (besides D'Eon et al. 2002) participated following an overnight fast. In fact, the lower glucose Ra and Rd in the study of D'Eon et al. (2002) with elevated oestrogen only, and with elevated oestrogen and progesterone compared to low oestrogen and progesterone condition, was reported only as a trend as this difference did not quite reach significance ($0.05<P<0.1$). Nonetheless, the ovarian hormone-induced reduction in glucose kinetics noted in the above studies is supposedly due to oestrogen's ability to impair hepatic gluconeogensis (Matute and Kalkhoff 1973). However, Horton et al. (2002) hypothesized that the effect of oestrogen on hepatic glucose output appears to only become noticeable when the exercise intensity is sufficient to increase the demands on glucose utilisation to above a certain "critical level". At this "critical level" the demand on endogenous glucose production is sufficiently elevated such that the effect of oestrogen suppression on gluconeogenesis is

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evident in a reduced glucose Ra. As illustrated by Horton et al. (2002) this theory is clearly supported by comparing the glucose kinetic results of Campbell et al. (2001), Horton et al (2002) and Zderic et al. (2001). When the subjects in the study of Zderic et al. (2001) exercised at approx. 50% VO_2max, glucose Ra was significantly lower in the ML phase vs. EF phase, with Ra averaging around 28.4 vs. 33.4 µmol/kg.min, respectively. However, no difference was noted between menstrual phases when these subjects exercised at only 45% VO_2max and glucose Ra averaged around 18-19 µmol/kg.min. Thus when there was less demand on endogenous glucose production, no menstrual phase effect was evident. In the study of Horton et al. (2002) subjects were slightly less trained than those in the study of Zderic et al. (2001) and so when at an equal exercise intensity of 50%VO_2max, the absolute workload was lower, thus demanding a lower total fuel utilisation and hence lower glucose utilisation and less demand on endogenous glucose production such that glucose Ra was at 19-21 µmol/kg.min (Horton et al. 2002). At this lower glucose Ra no difference was observed between the EF, MF and ML phases (Horton et al. 2002) as was similarly observed in the study of Zderic et al. (2001) at 45%VO_2max. Well-trained subjects in the study of Campbell et al. (2001) exercised at 70% VO_2max and thus increased the demand on endogenous glucose production above the “critical level” to supply sufficient glucose to fuel the high energy demand, causing a noticeable difference in glucose Ra between the EF and ML phase (~33 vs. 25 µmol/kg.min, respectively). However, when subjects in the study of Campbell et al. (2001) received an energy drink during exercise the difference in glucose Ra between menstrual phases was no longer significant as glucose Ra was now largely determined by exogenous glucose absorption. This theory proposed by Horton et al. (2002) can be extended to the condition of when subjects exercise only a few hours postprandially (but do not receive glucose supplements during exercise). Under these conditions it appears that the demands on glucose utilisation needs to exceed an even higher critical level before a difference in glucose kinetics between menstrual phases becomes evident. For example, the subjects in the study of D’Eon et al. (2002) exercised at 60%VO_2max following a 3-4 hours postabsorptive period and this exercise intensity resulted in a glucose Ra of 38.8-44.4 µmol/kg.min for the high oestrogen and high oestrogen plus progesterone conditions, compared to 47.2- 50 µmol/kg.min for the low oestrogen and progesterone condition. This tendency for a

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lower glucose Ra with supplemented ovarian hormones compared to non-supplemented condition did not quite reach significance. In contrast, in the study of Suh et al. (2002) subjects exercised three hours postprandially at 45% and 65% VO_2max and no noticeable difference was observed in glucose kinetics between the follicular and luteal phases, possibly because these intensities did not challenge glucose production sufficiently as noted by the lower glucose Ra values (27.7 and 41.7 μmol/kg.min for the 45 and 65%VO_2max trials, respectively) when compared to those reported in D'Eon et al. (2002). It would be interesting to challenge this hypothesis with a study where glucose kinetics is measured in subjects exercising at high intensities (greater than 70%VO_2max) following a short postprandial period in various menstrual phases.

Thus in summary, glucose kinetics appears to be influenced by menstrual phase when the energy demand of exercise is sufficiently high to pressurise endogenous glucose production. However, it appears that the postprandial period is a major determinant of the level of the demand on endogenous glucose production that is necessary before the influence of menstrual phase becomes evident. This can be explained as oestrogen or both oestrogen and progesterone impose a restriction on endogenous glucose production by suppressing gluconeogenesis, and with the scenario of exercise following a short postprandial period we could expect hepatic glycogenolysis to make a greater proportional contribution to endogenous glucose production than from gluconeogenesis relative to a condition that imposes a longer fasting period. Finally, when exogenous glucose is provided throughout exercise the influence of menstrual phase on glucose kinetics is negligible as this condition minimizes the demand on endogenous glucose production (Campbell et al. 2001).

Metabolic clearance rate (MCR) of glucose, calculated as the quotient of glucose Rd and plasma glucose concentration, is often reported to be lower with elevated oestrogen (Carter et al. 2001) or oestrogen and progesterone (Campbell et al. 2001); corresponding with ovarian hormone-induced reduction in glucose Rd and/or increase in plasma glucose concentration.

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In general, when the glucose kinetics are reduced in the presence of oestrogen or oestrogen and progesterone, the drop in glucose production could be attributed to a decrease in either, or both, hepatic glycogenolysis and gluconeogensis. As mentioned previously, both oestrogen and progeserone are known to inhibit hepatic gluconeogenesis (Matute and Kalkhoff 1973). However, oestrogen alone has been demonstrated to augment hepatic glycogenolysis during exercise in ovarectomised rats (Campbell and Febbraio 2002), but whether the same response occurs in humans remains indeterminate. The drop in glucose Ra with elevated ovarian hormones could also be a consequence of the decrease in glucose Rd and MCR. The decrease in glucose uptake and MCR in the LP or with ovarian hormone supplements may be related to a decrease in whole body carbohydrate utilisation as sometimes noted by a reduced RER and reduced total muscle glycogen utilisation, due to an increase reliance on lipid metabolism. The reduced glucose Rd reported during exercise in the luteal phase may be related to a reduced capacity for contraction-stimulated glucose uptake into quadriceps muscles (comprising mostly type I fibres) observed in the presence of progesterone even when progesterone is combined with physiological concentrations of oestrogen (Campbell and Febbraio 2002). However, oestrogen alone promotes contraction-stimulated glucose uptake into type I quadriceps muscle fibres (Campbell and Febbraio 2002) and thus does not agree with the reduced whole body glucose uptake reported in studies where exogenous oestrogen supplements were administered (Carter et al. 2001; D'Eon et al. 2002; Rudy et al. 1997).

1.2.1.3.6 Indirect estimation of muscle glycogen use

Glucose metabolic tracer studies often make the assumption that 100% of glucose uptake (Rd) is oxidised and therefore glucose Rd approximates plasma glucose oxidation rate. The difference between total carbohydrate oxidation estimated by indirect calorimetry (RER) and glucose Rd provides an estimate of muscle glycogen use during exercise. However, this is a crude assumption as the percentage of glucose Rd oxidised is probably closer to 70-90% and may vary depending on the study conditions and thus the calculation underestimates glycogen use and should be considered as minimal muscle glycogen utilisation. In addition, as previously discussed (under section 4.1.1) a caution is extended as to the use of RER values in determining substrate

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utilisation due to the possible influence of oestrogen in stimulating triacylglycerol resynthesis, which could elevate RER and factitiously overestimate carbohydrate utilisation (Ellis et al. 1994). Possibly for these reasons some studies measuring glucose kinetics have not estimated muscle glycogen use (Carter et al. 2001; Horton et al. 2002; Suh et al. 2002) and may explain why others have found no difference in estimated glycogen use between menstrual phases (Campbell et al. 2001) or with oestrogen treatment (Rudy et al. 1997). However, some studies which reported a lower glucose uptake (Rd) with elevated oestrogen (D'Eon et al. 2002) or oestrogen and progesterone (Zderic et al. 2001) also reported lower estimated muscle glycogen use during exercise under these conditions compared to EF phase conditions. Interestingly, D'Eon et al. (2002) found that although glucose uptake was suppressed with elevated oestrogen plus progesterone, estimated muscle glycogen use was in fact equal or greater than EF phase condition in which oestrogen and progesterone concentrations are low, resulting in a similar total carbohydrate use. Such a finding is contrary to reports from other authors whose muscle biopsy data suggest muscle glycogen sparing in the luteal phase with elevated oestrogen and progesterone concentrations (Hackney 1999). However, the rise in ovarian hormones in the study of D'Eon et al. (2002) was not natural, but was experimentally induced and during the oestrogen plus progesterone condition, oestrogen was elevated to within normal luteal phase levels. However, progesterone increased to around 151.4 nmol/L (or 47.6 ng/ml), which is higher than normal luteal phase levels. Therefore the muscle glycogen sparing that has been reported to occur in the luteal phase (Hackney 1999; Zderic et al.2001) must be largely due to the elevated oestrogen levels that occur during this phase and could possibly be more pronounced during the LF phase where oestrogen alone is elevated. Furthermore, D'Eon et al. (2002) described an interesting inverse correlation where free fatty acid concentration explained 50% of the variance in estimated muscle glycogen use, where FFA concentration was greater with oestrogen supplementation. This possibly infers that an oestrogen-induced increase in FFA availability promoted glycogen sparing during exercise (D'Eon et al. 2002). Therefore the influence of oestrogen and progesterone on muscle glycogen utilisation may depend on their influence on free fatty acid availability or oxidation.

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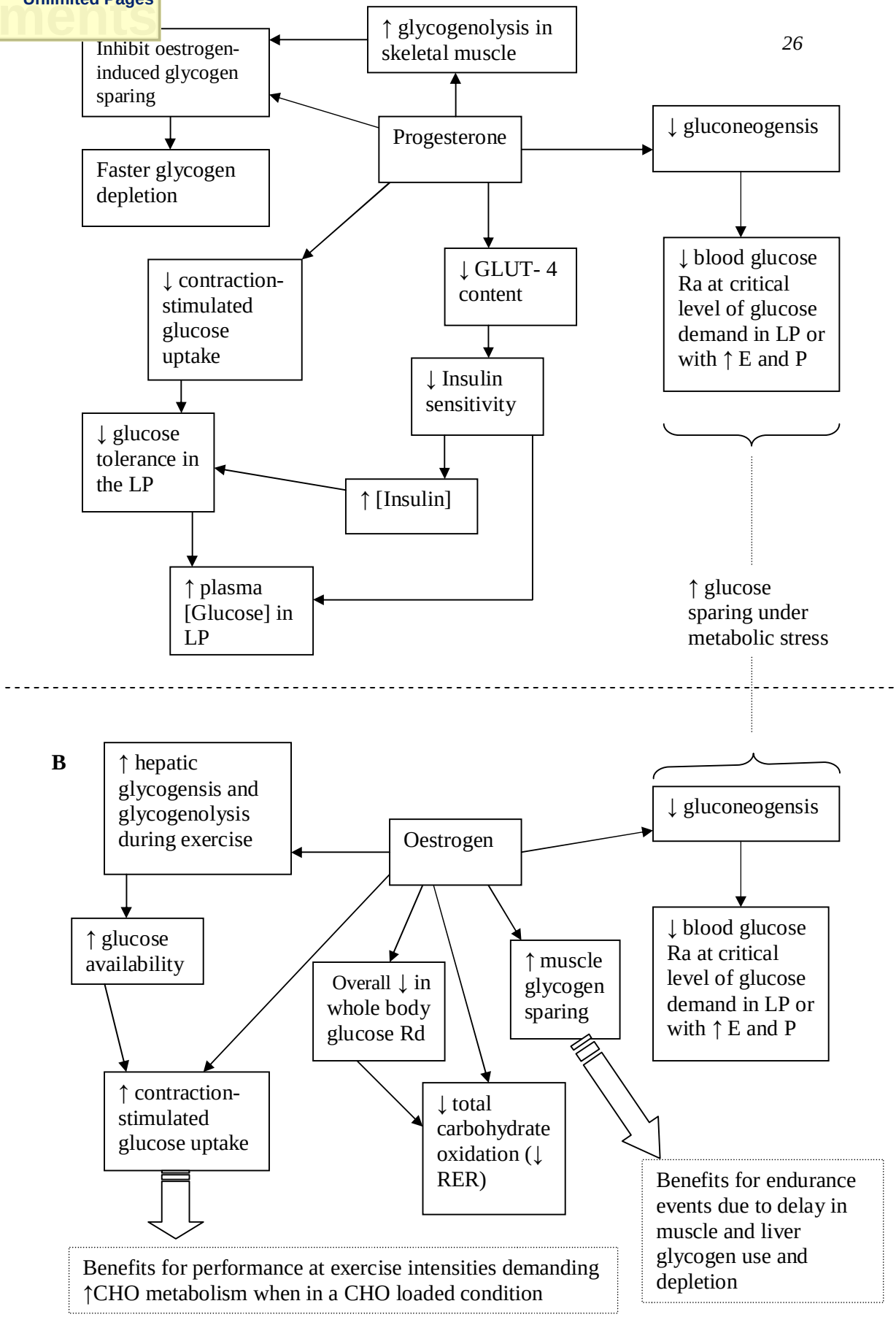

Figure 2. Summary of the effects of progesterone (A) and oestrogen (B) on carbohydrate metabolism. LP- luteal phase, E- oestrogen, P- progesterone, Ra- rate of appearance, Rd- rate of disappearance, RER- respiratory exchange ratio, CHO- carbohydrate

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1.2.1.4 Resultant effect of the ovarian hormones on various aspects of fat metabolism (Refer to Figure 3 and 4 for a flow diagram summary)

1.2.1.4.1 Plasma free fatty acid (FFA) concentration

Circulating FFA concentration tends to be increased in the presence of elevated oestrogen at rest and during submaximal exercise (D'Eon et al. 2002; Ruby et al. 1997). Surprisingly, some studies have observed lower FFA concentrations in the ML phase compared to the follicular phase during submaximal exercise (Campbell et al. 2001; Bonen et al. 1983). However, Campbell et al. (2001) only observed lower FFA levels in luteal phase when subjects exercised following an overnight fast and this observation disappeared when these subjects ingested a carbohydrate energy drink during exercise. Conversely, Bonen et al. (1983) only observed a decrease in FFA concentration in the ML phase in the group of subjects that received a glucose load and not in the fasted or control groups. Thus the conditions necessary to cause circulating FFA concentrations to differ between the luteal and follicular phases during exercise remains obscure. It is possible, however, that the elevated progesterone levels in the luteal phase may antagonise the oestrogen-induced increase in FFA availability as supported by the high progesterone concentration (~40 ng/ml) reported during the luteal phase in the studies of Bonen et al. (1983) and Campbell et al. (2001) that would substantially reduce the E/P ratio. Other authors however, have reported no difference between resting and exercising FFA concentration between menstrual phases (Horton et al. 2002; Lavoie et al. 1987; Nicklas et al. 1989).

1.2.1.4.2 Plasma glycerol concentration

Changing blood glycerol levels is used as a rough indication of lipolysis. On hydrolysis of a triacylglcerol molecule glycerol is almost always released into the bloodstream; a hydrolysed glycerol molecule must be rephosphorylated in the liver before it can be used in the re-esterification of FFAs into a new triacylgylcerol molecule. However, glycerol can also be used as an oxidisable fuel if incorporated into the glycolytic pathway.

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Most studies have found no difference in glycerol concentration at rest or during exercise between different menstrual phases (Bonen et al. 1983; Horton et al. 2002; Lavoie et al. 1987; Zderic et al. 2001) or in men receiving either placebo or oestrogen supplements (Carter et al. 2001). Only Campbell et al. (2001) found glycerol levels to be significantly higher in the follicular phase compared to the luteal phase during exercise following an overnight fast. Thus in the study of Campbell et al. (2001) lipolysis during prolonged submaximal exercise may have been stimulated to a greater extent in the follicular phase as suggested by the observation of both elevated glycerol and FFA levels. However, as mentioned earlier, the findings of Campbell et al. (2001) may be a consequence of the very high progesterone concentrations in their subjects' LP and therefore also a low E/P ratio. Thus their results do not indicate enhanced lipolysis in the FP but rather suppressed lipolysis in the LP in this particular case and not necessarily in general.

1.2.1.4.3 Systemic glycerol kinetics

Determination of glycerol Ra by tracer methodology is routinely used as a more precise index of whole body lipolytic rate (Wolfe 1992). Again, this is based on the assumption that following triacylglycerol hydrolysis in muscle and adipose tissue, glycerol must be released into the blood. Glycerol must be rephosphorylated by the enzyme glycerol kinase, present only in the liver and to a lesser degree in the kidneys, before it can be re-used in triacylgycerol reesterification. Therefore, it is assumed that hepatic clearance of glycerol from the blood is the only significant route of irreversible loss of a glycerol tracer (Wolfe 1992). However, this assumption has been challenged as some authors (Landau et al. 1996) have found that only half of the glycerol Ra is taken up by the splanchnic bed and therefore the periphery must be taking up the rest. Secondly the findings of others (Elia et al. 1993) suggests that muscle may metabolise a significant amount of glycerol and therefore not all of the glycerol released by intramuscular triacylglycerol hydrolysis will appear in the blood stream.

Nonetheless, only two studies to date have investigated the influence of oestrogen on glycerol Ra/Rd by administering oestrogen supplements to amenorrhoeic female athletes (Ruby et al. 1997) or to men (Carter et al. 2001) and both studies reported

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unaltered glycerol kinetics at rest and during exercise as a result of oestrogen treatment. However, a gender difference during exercise has been reported, with glycerol Ra being higher in women (in their follicular phase) than in men who were matched for fitness, age and body composition (Mittendorfer et al. 2002). Glycerol Ra was 30% higher in women than in men during exercise at 50% VO_2peak and the total lipolytic response (estimated as the integrated area under the glycerol Ra curve) was 60% greater in women than in men. This gender difference in glycerol kinetics is supported by the findings of Carter et al. (2001b) and Friedlander et al. (1999). The increased lipolytic response to exercise in the women was not associated with a variation in the catecholamine response (Mittendorfer et al. 2002). Oestrogen has been reported to increase beta-adrenergic receptor sensitivity in adipose tissue (Beniot et al. 1982). That is, Beniot et al. (1982) reported a heightened sensitivity to catecholamines in oestrogen-supplemented rats with corresponding increase in hormone sensitive lipase activity. These authors suggest that oestrogen acts via its catechol-oestrogen derivative to potentiate the lipolytic action of epinephrine by competing with catecholamines for catechol-O-methyltransferase (Benoit et al. 1982). However, gender comparisons report no difference in lipolytic sensitivity to beta adrenergic stimulation (Jensen et al. 1996; Millet et al. 1998); albeit in women using oral contraceptives, who experience low concentrations of endogenous oestrogen or where high doses of catecholamines were administered masking differences that may be apparent at lower concentrations, such as those experienced during submaximal exercise. In addition, the observed gender variation in lipoytic response may be related to variation in alpha adrenergic receptor activity (Mittendorfer et al. 2002). That is, the lipolytic rate during exercise is modulated in men by stimulation of alpha adrenergic receptors whereas alpha adrenergic receptor activity does not appear to regulate lipolysis during exercise in women (Hellström et al. 1996). Thus the lipolytic response to oestrogen in men (Carter et al. 2001) may be different to women.

More recently, glycerol kinetics have been compared at rest and during submaximal exercise in eumenorrhoeic women in their EF and ML phase and then after four months of oral contraceptive supplementation (Casazza et al. 2004). No significant difference was found between menstrual phases in a sub-sample of n=5, but oral contraceptive use increased glycerol Ra during submaximal exercise (n=8). Oral contraceptive use also

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resulted in higher cortisol concentration and this is presumably causative of the heightened lipolytic rate (Casazza et al. 2004). Nonetheless, the menstrual phase effect on glycerol flux during exercise should be studied further by considering the influence of the E/P ratio in the ML phase and including the LF phase in the comparison as the lipolytic effects of oestrogen may be antagonised by the coincident increase of progesterone in the ML phase.

1.2.1.4.4 Free fatty acid kinetics and the contribution of various lipid sources as metabolic fuel

Free fatty acid Ra provides an index of plasma free fatty acid availability and measures the release of fatty acids that are primarily derived from the hydrolysis of adipose tissue triacylglycerol into plasma (Mittendorfer et al. 2002). When used as a measure of lipolytic response, FFA Ra does not account for triacylglycerol re-esterification (Romijn et al. 2000). Free fatty acid Rd measures the rate of uptake into tissues and has been used as an estimate of plasma FFA oxidation rate (Romijn et al. 1993); albeit a crude estimate as the actual proportion of FFA uptake that is oxidised can vary and has been reported to be as low as 50% (Friedlander et al. 1998).

Until recently only one study had considered the influence of menstrual phase on free fatty acid kinetics at rest with induced hypoinsulinemia following an overnight fast (Heiling and Jensen 1992). Such an intervention was imposed in order to maximise lipolytic stimulation. No difference was observed between the follicular and luteal phase under these conditions (Heiling and Jensen 1992). A further study has attempted to identify the influence of oestrogen on lipolysis by comparing palmitate kinetics between oestrogen deficient and oestrogen supplemented postmenopausal women (Jensen et al. 1994). Initial palmitate flux was similar between treatments but differed between conditions following the induction of a hormonal clamp that was imposed in an attempt to isolate the actions of oestrogen without the influence of other hormonal regulators of lipolysis. Palmitate release was greater in the oestrogen deficient versus oestrogen-supplemented condition, indicating greater lipolytic rate with low circulating oestrogen levels (Jensen et al. 1994). However the imposed GH and insulin clamp may have

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induced a factitious scenario that does not reveal the holistic lipolytic influence of oestrogen, which should include its modulation of these lipolytic-regulating hormones (refer to Section 1.2.1.2). A recent study compared resting dietary FFA uptake into various body stores between genders and between the follicular and luteal phase of women (Uranga et al., 2005). There were no significant menstrual phase specific variations, but a high variability within the women was observed.

A number of gender comparison studies have measured FFA flux (Burguera et al. 2000; Friedlander et al.1999; Mittendorfer 2002; Romijn et al. 2000) but have produced inconsistent results. When women and men were matched for body composition (with respect to percent body fat), training status and age, the women had a 70% greater increase in total FFA availability (Ra) during exercise at 50% VO_2peak despite a similar total FFA oxidation rate. The greater plasma FFA availability in the women resulted in greater plasma FFA oxidation (due to an increased Rd) while the men favoured non plasma FFA oxidation (Mittendorfer et al. 2002). Non plasma FFA oxidation may refer to a combination of intramuscular triacylglycerols (IMTG), very low density lipoprotein triacylglycerols (VLDL-TG) and interstitial lipid. However, despite similar percent body fat, actual fat mass and fat free mass was greater in the males than females due to a difference in total body mass. Nonetheless the observed gender differences were maintained when expressed relative to fat free mass. Contrary to the findings of the former study, correcting for lean body mass in the study of Romijn et al. (2000) resulted in the (apparent) difference in FFA kinetics between genders to be no longer significant during exercise at various intensities. Likewise, Burguera et al. (2000) found no gender difference between systemic and leg FFA kinetics at rest and during 90 min of exercise at 45% VO_2max; albeit large differences in body composition between subject groups. Friedlander et al (1998) studied the effect of endurance training on substrate kinetics in women in their mid-follicular phase. Using glycerol and palmitate tracers they found that training led to an increased capacity to mobilise adipose tissue stores accompanied by an increased total fat utilisation mainly due to an increased capacity for plasma FFA uptake and oxidation. Friedlander et al (1999) found that where women demonstrate a significant training-induced substrate shift towards fat utilisation, the male subjects did not alter their proportional substrate selection due to training. In addition the men used more carbohydrates as a fuel source compared to the women when exercising at an

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equal relative intensity. However, despite the distinct differences in substrate utilisation, FFA kinetics was not significantly different between genders at rest or during exercise (Friedlander et al. 1999). Therefore the majority of gender studies report FFA flux to be similar between men and women at rest and during submaximal exercise.

A gender study on intramuscular triaylglycerol storage and utilisation measured by muscle biopsy before and after exercise (60%VO_2max for 90 min) revealed that women had a higher resting intramuscular triacylglycerol content than the men in the study and used 25% of resting stores during exercise, while the intramuscular triacylglycerol content was not changed due to exercise in the men (Roepstorff et al. 2002; Steffensen et al. 2002). These studies were supported by a previous study where glycerol Ra was measured by arteriovenous difference across exercising leg muscle to provide a measure of intramuscular lipolysis and hence IMTG use (Bergman et al. 1999); but this technique is limited as it does not allow for the possible use of hydrolysed glycerol as a glycolytic substrate within the muscle. Nonetheless, by application of this method, measured release of glycerol across working leg muscle produce negligible values in men, suggesting no significant IMTG lipolysis during moderate intensity exercise (Bergman et al. 1999). However, these gender differences may not be directly related to variation in oestrogen concentration but rather phenotypic differences in fibre type, hormone sensitive lipase (HSL) activity and heightened sensitivity of HSL to potential stimulators (such as epinephrine) (Steffensen et al. 2002). Others suggest that the gender-difference observed by Steffensen et al. (2002) regarding IMTG use, is simply due to the greater fat mass to lean body mass ratio in women and the reduced resting fat oxidation rate in women that promotes greater IMTG storage and hence utilisation in exercise (van Loon 2004).

These findings are not supported by studies that estimate intramuscular triacylglycerol use during exercise from a combination of isotopic tracer and indirect calorimetry techniques; where plasma FFA oxidation is subtracted from whole body fat oxidation in order to estimate IMTG oxidation (which by this methodology is frequently termed “non plasma FFA oxidation” as no distinction can be made between energy derived from IMTG and VLDL-TG oxidation). For example, Mittendorfer et al. (2002) found that for total fat oxidation during exercise at 50%VO_2max for 90 min, non plasma FFA

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provided 54% of fat source in men, while non plasma FFA only provided 24% of the fat source in women. Conversely, another tracer study found that non plasma FFA supplied between 65 to 80% of total FFA oxidation in women when exercising at 45 and 65% VO_2max (Friedlander et al.1998). (The percentage contribution of non plasma FFA to fat oxidation is likely to be overestimated in this latter study, as an acetate correction factor was not applied in the calculation of plasma FFA oxidation to account for retention of the tracer in exchange reactions of the tricarboxylic acid cycle (refer to section 3.2 and 3.3)). However, the validity of the indirect estimation of IMTG use by tracer and indirect calorimetry has recently been questioned (Bergman et al. 1999, Roepstorff et al. 2002), where this technique has been compared to muscle biopsy and arteriovenous concentration difference measurements. Results suggest that the indirect estimation of IMTG use is grossly overestimated specifically in male subjects. This can be understood when considering the shortfalls of the assumptions which support the estimate of IMTG oxidation from FFA tracer and indirect calorimetry data. FFA tracer dilution methodology considers energy production sourced from VLDL-TG to be negligible, where in fact recent findings suggest that VLDL-TG oxidation provides between 5 to 25% of total energy production depending on diet and training status (Kiens 2006). Furthermore, plasma FFA Rd during exercise is assumed to equal plasma FFA oxidation, when in fact only 80 to 96 % of FFA uptake during exercise is oxidised (Kiens 2006) and a large portion of FFA uptake occurs in inactive fibres or muscles, some of which is reesterified and incorporated into IMTG stores (Schrauwen-Hinderling et al. 2003; Kiens 2006); thus further limiting the estimation of IMTG use from FFA tracer and indirect calorimetry methodology. Muscle biopsy protocols, however, can only provide overall concentration of the intracellular metabolite and do not take into account the intracellular kinetics. For example, Guo et al. (2000) demonstrated simultaneous hydrolysis and synthesis of IMTG during exercise (45%VO_2peak) resulting in no net difference in IMTG content post exercise (measure by muscle biopsy) despite approximately 50% contribution of IMTG oxidation to total fat oxidation (measured by pulse-chase technique, where the IMTG pool was pre-labelled prior to exercise). Moreover, measurement of IMTG content from a biopsy sample can be highly variable due to the high variability of IMTG concentration within muscle samples, the possibility of inadvertently including extramyocellular lipid droplets when

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extracting a muscle sample and due to the likelihood of including different proportions of type I to II fibres in repeated samples (Watt et al.2002). Most of the inconsistency in the literature regarding IMTG use based on muscle biopsy arose from studies using wet muscle sample dissection and biochemical extraction procedures. Recent freeze dried sample techniques and histochemical Oil red O-staining have consistently produced findings of significant IMTG use in both male and female subjects (Kiens 2006; van Loon 2004). Furthermore Kiens (2006) suggests that protocols employing only short exercise durations are more likely to overlook small but significant changes, as triacylglycerol molecules are very energy dense and small amount provide large amounts of energy. Recent studies using proton-magnetic resonance spectrometry (^{1}H-MRS) confirm the reliance on IMTG oxidation during moderate-intensity endurance exercise in both men and women (van Loon 2004). Thus gender and menstrual phase comparative studies using the latest techniques for IMTG quantification are encouraged.

Of the primary hormonal regulators of lipolysis, Steffensen et al. (2002) reported higher plasma insulin and lower epinephrine concentration at rest and during 90 min of exercise (60%VO_2peak) in women compared to men (including large sample groups of n=21). Carter et al. (2001b) also found epinephrine concentration to be lower in females than in males after 90 min of exercise (60% VO_2peak) but found no gender difference in norepinephrine or insulin concentration. Other studies, however, have not identified gender differences in these lipolytic regulators (Burguera et al. 2000; Mittendorfer et al. 2002; Roepstorff et al. 2002). Interestingly, a recent study has found insulin to stimulate fatty acid transport by increasing the fatty acid translocase, FAT/CD36 protein content and had no affect on plasma membrane bound fatty acid binding protein (FABPpm) (Chabowski et al. 2004). This is of particular interest considering the previously mentioned effect of oestrogen and progesterone on insulin. In addition, Kiens et al. (2004) have reported 49% higher levels of FAT/CD36 protein in women than in men irrespective of training status, while no gender difference was noted in FABPpm. Although it may be possible that such gender difference could account for the observed difference in IMTG content and hence IMTG utilisation during exercise, it does not explain differences noted in plasma FFA oxidation as most studies demonstrate similar FFA uptake during exercise between genders. Besides, Roepstorff et al. (2004) has found that transsarcolemmal transport is not a limiting factor for plasma long chain fatty

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acid (LCFA) oxidation but instead suggests that plasma LCFA oxidation is limited by intracellular regulatory mechanisms. Nonetheless the fatty acid translocase (FAT/CD36) as a possible point of variation in fat metabolism between menstrual phases cannot be excluded due to previous reports of menstrual phase differences in insulin concentration. Animal models support the concept of oestrogen increasing FFA uptake into skeletal muscles (Ellis et al. 1994; Rooney et al. 1993) and thus suggests the possibility of variations in FFA uptake between menstrual phases.

Roepstorff et al. (2004) explains that intracellular sites of regulation of plasma LCFA oxidation rate possibly occurs at the point post cellular uptake, where LCFA are either channelled into esterification or beta oxidation, or at the point of entry of LCFA into the mitochondria or both. The entry of LCFA into the esterification pathway depends on the activity of AMP-activated protein kinase (AMPK) that inhibits the enzyme (glycerol-3-phosphate acyltransferase) that initiates triacylglycerol synthesis. Since the activity of AMPK is inversely related to muscle glycogen content, the lower the muscle glycogen content the greater the activity of AMPK and less esterification and more beta-oxidation of LCFA (Roepstorff et al. 2004). Entry of LCFA into mitochondria during exercise has been shown to be less dependent on changes in malonyl CoA concentration- which is a regulator of the carnitine palmitoyltransferase I transporter; and is rather related to muscle free carnitine concentration (Roepstorff et al. 2004). Whether the ovarian hormones can be implicated in altering these regulatory systems and so account for previously reported menstrual phase differences in total body fat use in exercise or gender differences in plasma FFA oxidation is indeterminate.

Most gender comparative studies run the experimental trials during the follicular phase of their female subjects when the ovarian hormones are relatively low, although oestrogen concentration may still be higher than the circulating levels in the male group (Steffensen et al. 2002). Nonetheless, no evidence exists to attribute the observed gender differences directly to variations in circulating ovarian hormones. The current literature is lacking in studies that compare plasma FFA kinetics and oxidation between conditions of varying ovarian hormone concentrations as occurs naturally over the course of the menstrual cycle. Thus, the second study presented in this thesis (Chapter 3) assessed plasma FFA kinetics with the use of stable tracers during submaximal

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exercise in women across the menstrual cycle and considered the influence of the E/P ratio on these metabolic variables. Coincidently, however, in a very recent publication, FFA kinetics and oxidation was compared between the EF and ML phase during rest and submaximal exercise and then again after 4 months of oral contraceptive use (Jacobs et al. 2005). No difference was observed between the EF and ML phase in any of the measured variables. Oral contraceptive use also failed to alter FFA kinetics and oxidation, but did increase the rate of FFA reesterification (Jacobs et al. 2005). However, due to the accumulated evidence for oestrogen and progesterone to act antagonistically when influencing various aspects of metabolism, in the study presented in this thesis (Chapter 3), instead of a simple between menstrual phase comparison, of for example EF vs. ML, we focused on the influence of the E/P ratio on FFA kinetics. Thus the aim was to identify whether the interactive effect of oestrogen and progesterone in the ML phase will determine the overall response, which may be overlooked in a straight-forward EF vs. ML comparison.

1.2.1.4.5 Animal studies and fat metabolism

Ovarectomy reduces the activity of key enzymes in fat metabolism, namely carnitine palmitoyltransferase I (CPT I) and beta-3-hydroxyacyl-CoA dehydrogenase (β–HAD). (Campbell and Febbraio 2001). Oestrogen restores the activity of these enzymes, while progesterone inhibits these positive actions when oestrogen is at physiological concentrations (Campbell and Febbraio 2001). A supraphysiological concentration of oestrogen overrides the negative effects of progesterone (Campbell and Febbraio 2001). This rat model therefore demonstrates the ability of the ovarian hormones to alter the capacity for skeletal muscle to oxidize FFAs by directly impacting on the cellular metabolic pathways. The proposed mechanism for these observations is an oestrogen-induced up-regulation of the gene transcription and hence elevated protein levels of these enzymes (Campbell and Febbraio 2001). Previous studies have reported the presence of oestrogen and progesterone receptors in skeletal muscle (Kahlert et al. 1997). Interestingly, no difference was observed in the activity of the key enzyme of the tricarboxylic acid cycle (TCA)- citrate synthase, as a consequence of ovarectomy, oestrogen, progesterone, or a combination of oestrogen plus progesterone treatment at physiological concentrations (Campbell and Febbraio 2001).

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In another rat study it has been demonstrated that oestrogen has an active role in promoting fat metabolism by increasing lipid availability to skeletal and cardiac muscle (Ellis et al. 1994). Male rats received oestrogen treatment and this reduced adipocyte lipoprotein lipase (LPL) activity and increased muscle LPL activity, thus promoting a redistribution of lipids from adipose to muscle tissue, thereby increasing muscle uptake of plasma triacylglycerol-FFAs and elevating resting intramuscular FFA content and resting and post-exercise muscle triacylglycerol content. In particular, oestrogen promoted triacylglycerol esterification during submaximal exercise in the red vastus muscle as triacylglycerol content was greater post exercise than at resting levels. Previous studies have demonstrated an increase in liver synthesis and secretion of VLDL-TG with oestrogen administration, which would contribute to the oestrogen-induced increase in lipid availability (Kenagy et al. 1981). In addition others have also demonstrated an increase in lipolysis and reduced fatty acid synthesis in isolated fat cells from oestrogen treated rats, while progesterone had no effect compared to control/unsupplemented rats (Hansen et al. 1980). However, we cannot exclude the possibility of a gender difference in the response to oestrogen treatment, as the receptor population of the endogenous sex hormones are reportedly different between genders (Haffner et al. 1995). In addition, interspecies differences may occur in the regulation of lipid metabolism (Spriet 1998).

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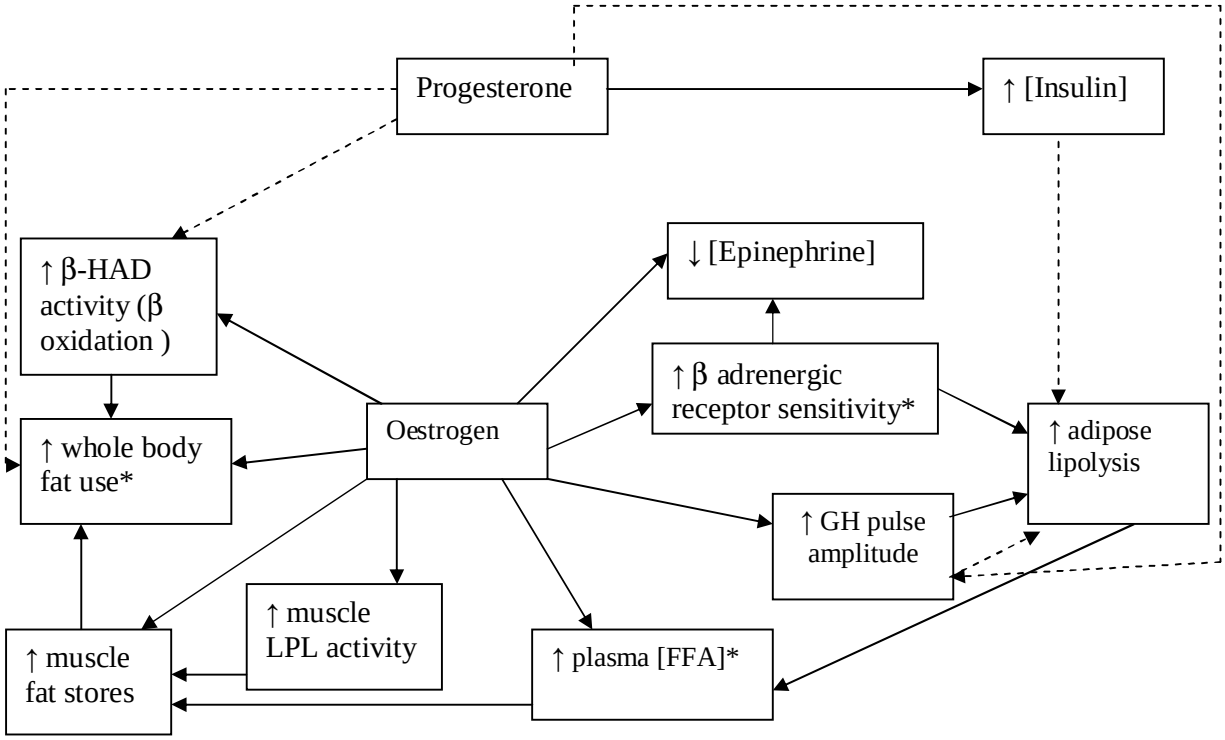

Figure 3. Summary of the proposed influence of oestrogen and progesterone on fat metabolism during submaximal exercise as supported by most published observations. Boxes marked with * indicate that not all published studies agree. A solid line (⟶) indicates stimulation whereas a dashed line (⇢) indicates inhibition of the details in a box. [ ] denotes concentration, β-HAD denotes beta-3-hydroxyacyl-CoA dehydrogenase, LPL refers to lipoprotein lipase, FFA refers to free fatty acid, GH refers to growth hormone.

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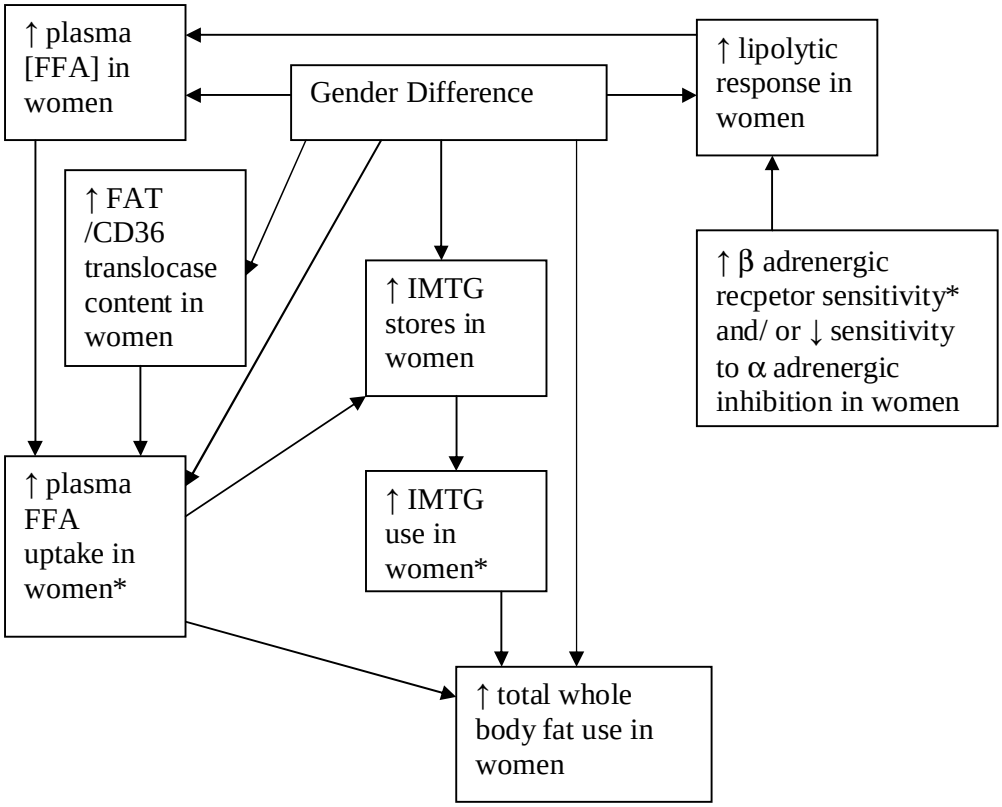

Figure 4. Summary of gender variation in fat metabolism during exercise. Boxes marked with * indicates that some reports do not agree. FFA refers to free fatty acid, IMTG refers to intra muscular triacylglycerol.

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1.2.1.5 Influence of ovarian hormones or gender on protein metabolism

Bailey et al. (2000) found the concentration of various amino acids (namely, alanine, glutamine, proline and isoluecine) to be lower in the ML phase compared to the EF phase at rest and during prolonged submaximal exercise in subjects exercising 3 hours postabsorptively. This suggests greater amino acid catabolism during exercise in the luteal phase. The amino acid concentration difference between phases was smaller if a carbohydrate supplement was ingested during exercise compared to a placebo drink (Bailey et al. 2000). Similarly, Lamont et al. (1987) found protein catabolism to be greater during the ML phase compared to the EF phase measured by total urea nitrogen excretion over four days including a 60 min period of exercise at 70%VO_2max. Total urinary urea nitrogen excretion over the exercise period was also greater in the ML phase compared to the EF phase (Lamont et al. 1987). This finding was confirmed by a subsequent isotopic tracer study that reported 12% greater leucine flux and 24% greater leucine oxidation, but no difference in non-oxidative leucine disposal (i.e. protein synthesis) at rest in the mid-luteal phase compared to mid follicular phase in eumenorrhoeic women; unfortunately an exercise period was not included in the study (Lariviere et al. 1994). However, it must be noted that although the use of L[1-13C]leucine is considered the reference method for estimating whole body protein metabolism in most physiological conditions, there are many examples in the literature which demonstrate that by using different amino acid tracers different experimental outcomes are attained (Wagenmakers 1999). Thus, it appears that the dynamics are amino acid specific and cannot necessarily be extrapolated to all amino acids or whole body protein metabolism. Leucine is a branch chain amino acid that is preferentially catabolised by muscle when energy is sourced from protein and thus when extrapolating leucine dynamics to whole body protein metabolism, protein oxidation is overestimated, protein synthesis is underestimated and protein balance appears negative (Wagenmakers 1999). Nonetheless, menstrual phase comparative or gender studies employing the L[1-13C]leucine method have provided interesting insights, even if the findings are considered to reflect mainly branch chained amino acid dynamics rather than whole body protein metabolism, particularly during exercise as knowledge concerning the

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availability and utilisation of substrates sourced for muscle activity is of prime relevance.

Gender studies have found men to oxidise significantly more protein than women in their follicular phase during moderate exercise (Lamont et al. 2001; McKenzie et al. 2000; Phillips et al. 1993). While amino acid (leucine and lysine) flux, used as a measure of overall protein breakdown, was similar during exercise between genders in one study (Lamont et al. 2001) it was found to be greater in men than women in another study (McKenzie et al. 2000). Nonetheless, leucine oxidation was roughly 20-70% (Lamont et al. 2001; McKenzie et al. 2000; Phillips et al. 1993) greater in men than in women during exercise and provided on average 2% of energy expenditure in women and 3% of energy expenditure in men (Lamont et al. 2001; Phillips et al. 1993). In addition to the greater total and proportional protein use in the male subjects, all of these gender comparative studies also reported greater total and proportional carbohydrate oxidation in the males during exercise, while the females relied on a greater proportional contribution from lipid oxidation as estimated from RER (Lamont et al. 2001; McKenzie et al. 2000; Phillips et al. 1993). Changes in protein synthesis measured as non-oxidative leucine disposal (NOLD) also differed between studies. NOLD decreased during exercise but was significantly greater in women than in men (Lamont et al. 2001). Another study, however, found NOLD to remain similar between rest and exercise and found no differences between genders, however in that study subjects followed a diet that limited protein intake, and so resulted in a negative nitrogen balance for these endurance athletes, thus limiting protein synthesis (Phillips et al. 1993).

The gender difference in leucine oxidation is not associated with similar differences in muscle branched-chain 2-oxoacid dehydrogenase (BCOAD) activity (key mitochondrial enzyme for branched chain amino acid oxidation), or the proportion of activated BCOAD between genders (McKenzie et al. 2000). BCOAD is activated by dephosphorylation and the proportion of BCOAD that is activated is correlated with decreasing phosphorylating potential, and reduced pH and/or low glycogen concentration. As some studies have suggested glycogen storage, or use during exercise

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to differ between menstrual phases, we cannot exclude the possibility for a difference in protein use between menstrual phases to be related to differences in BCOAD activation.

To further understand the gender difference in leucine metabolism, it's regulation by beta adrenergic receptor stimulation was evaluated by imposing a beta adrenergic receptor blockade (Lamont et al. 2003). Leucine oxidation and Lysine Ra was increased in response to beta blockade in men, but not in women. In addition, carbohydrate utilisation increased in response to beta blockade but more so in men than women. The women increased FFA availability and relied to a greater extent on fat metabolism even under the imposed beta blockade. Therefore a gender difference exists in metabolic compensation to conditions of fuel restriction or increased energy demand (Lamont et al. 2003). Thus amino acids become an important fuel for men, whereas women are capable of continuing their heightened reliance on fat metabolism, possibly due to greater beta adrenergic receptor sensitivity to stimulation in the adipose tissue and hence greater lipolytic responsiveness (Lamont et al. 2003). This is supported by previous studies that have reported lower catecholamine response to exercise in women than in men (Carter et al. 2001b; Horton et al. 1998; Steffensen et al. 2002). The decreased need for protein oxidation and the greater fat oxidation during exercise in women may also be a consequence of the suggested increased cellular capacity for fat oxidation previously discussed.

1.2.2 Other non-reproductive physiological actions of the ovarian hormones

1.2.2.1 Ventilation

Pregnancy is known to be associated with an increase in ventilation. Furthermore, the luteal phase is also characterised by greater resting ventilation than in the follicular phase (Schoene et al. 1981; Williams and Krahenbuhl 1997). Progesterone is implicated for the increases in resting minute ventilation (V_E) (Bayliss and Millhorn 1992). Ventilation is under central autonomic control via the medullary inspiratory neurons, whose rate of firing and quiescence is determined by input from the pons, pulmonary stretch receptors, central and peripheral chemoreceptors, as well as receptors in joints and muscles (Vander et al. 1996). Progesterone influences this system via central receptors in the hypothalamus (Bayliss and Millhorn 1992). How the stimulation of

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hypothalamus-based progesterone receptors leads to stimulation of inspiratory motor neurons in the medulla oblongata remains unknown (Bayliss and Millhorn 1992). Furthermore, Bayliss and Millhorn (1992) have demonstrated how oestrogen promotes the induction or synthesis of progesterone receptors in the hypothalamus and is clearly related to the progesterone-induced increase in ventilation. The ability of oestrogen to augment the progesterone-stimulation of ventilation is apparent when considering the findings of Bonekat et al. (1987), where males received medoxyprogesterone acetate (MPA), which has fifteen times the progestational activity of natural progesterone, yet the males only responded with a sub-level increase in ventilatory drive over their placebo treatment when compared to the magnitude of response from follicular to luteal phase in eumenorrhoeic women. That is, the progesterone together with oestrogen as in the luteal phase produces a greater response than progesterone alone. Progesterone also increases ventilation by increasing chemosensitivity as demonstrated by an increased ventilatory response to hypoxia and hypercapnia as observed during the luteal phase compared to follicular phase (Dombovy et al. 1987; Schoene et al. 1981) or in men treated with medoxyprogesterone acetate (Bonekat et al. 1987). However, this is not consistently reported, as a recent study did not find an increase in chemosensitivity in the mid-luteal phase compared to the early follicular phase (Beidleman et al 1999). Furthermore, an increase in metabolic rate as reflected by an increase in body temperature is also known to elevate $V_{E.}$ Thus the elevated body temperature associated with the luteal phase may also contribute to the observed hyperventilation (Schoene et al. 1981).

The effect of elevated progesterone levels on minute ventilation during exercise remains equivocal. A number of studies have observed an increase in the ventilatory equivalent (the ratio of V_E to VO_2) or a decrease in the end tidal partial pressure of carbon dioxide during incremental exercise to exhaustion in the luteal phase versus the follicular phase or with MPA supplementation in males versus control condition (Bonekat et al. 1987; Dombovy et al. 1987; Jurkowski e al. 1981; Schoene et al. 1981). However, others have found no difference in V_E during maximal (Bemben et al. 1995; Lebrun et al. 1995) or submaximal exercise (Beidleman et al. 1999; De Souza et al. 1992; Hackney et al. 1991; Jurkowski et al. 1981). Williams and Krahenbuhl (1997) found minute ventilation to be significantly greater in the ML versus EF phase during 5 min of submaximal exercise at

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55% and 80% VO_2max, but no difference between other menstrual phases (i.e. late follicular, early luteal or late luteal). They attribute previous inconsistencies regarding the influence of menstrual phase on exercising ventilation to be a consequence of not regulating the timing of testing in each menstrual phase tightly enough.

Beidleman et al. (1999) viewed a progesterone-induced increase in V_E to be a potential benefit for athletes when exercising at altitude by possibly causing an increase in oxygen delivery to muscle. However, they found no improvements in VO_2max or endurance capacity at altitude, despite a small increase in oxygen saturation (3%) in the mid-luteal phase compared to the early follicular phase (Beidleman et al. 1999). Others view an increased ventilatory response to hypoxia and hypercapnia or to exercise to be deleterious for athletic performance due to the heightened sensation of dyspnoea that could limit exercise performance (Lebrun 1993). In this regard, Beidleman et al. (1999) reported a near significant correlation of VO_2max and time to exhaustion, with the perception of respiratory distress or fatigue, albeit no difference in these indicators between menstrual phases. Schoene et al. (1981) is the only study to report a difference in exercise performance with a coincident increase in V_E in the luteal phase versus follicular phase when they observed a shorter time to exhaustion during a maximal ramp test in the luteal phase (in non athletes only). However, an elevated V_E during exercise in the luteal phase in endurance athletes did not compromise maximal time to exhaustion (Schoene et al. 1981). Therefore the negative effects of an increase in V_E during exercise due to progesterone can be overcome with training as a result of a reduced sensitivity to “respiratory distress”. In summary, progesterone may alter V_E between menstrual phases, but based on current literature this action does not have a major effect on VO_2max, high intensity or submaximal endurance capacity.

However, it may be worthwhile considering whether a persistently higher respiratory rate over prolonged endurance events could contribute to a significant increase in metabolic demand and possibly contribute towards fatigue. Furthermore, despite some studies claiming to observe no change in ventilatory parameters during exercise between menstrual phases, the possibility of large inter-subject variability in ovarian hormones on the testing day and hence variable responses between the menstrual phases in each subject to obscure the influence of the ovarian hormones on ventilation during

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exercise needs to be considered. Thus, in the study presented in Chapter 5, we tested for significant correlations between the change in ventilatory parameters between menstrual phases during prolonged submaximal exercise and the ovarian hormone concentrations or the E/P ratio. Furthermore, we considered the influence of possible prolonged increases in respiratory drive coincident with increases in progesterone on metabolic demand.

1.2.2.2 Thermoregulatory system

An increase in basal body temperature by 0.3 to 0.5 degrees Celcius post ovulation is well documented and is attributed to the increase in progesterone concentration during the luteal phase. The increase in core temperature is due to an increase in the thermoregulatory set point that is brought about by progesterone acting centrally on the preoptic area (Janse de Jonge 2003). Oestrogen is believed to decrease body temperature, also by acting centrally and this is occasionally observed by in body temperature coincident with the pre-ovulatory surge in oestrogen. Therefore it is possible that the body temperature increase post ovulation is dependent on the ratio of oestrogen to progesterone. Horvath and Drinkwater (1982) investigated the response to exercise during the early follicular, ovulation and luteal phases under three ambient temperature conditions. However, only light exercise (30%VO_2max) was performed and only a small sample size was employed (n=4). They found no major thermoregulatory differences between menstrual phases during the various hot ambient temperature conditions. Conversely, a number of studies have found the resting temperature difference between the follicular and luteal phase to persist during exercise (Hessemer et al. 1985; Pirvanik et al. 1992). Therefore it is not surprising that the threshold temperature for the onset of sweating and cutaneous vasodilation is elevated in luteal phase (Hessemer et al. 1985; Pirvanik et al. 1992). In addition, the thermosensitivity during exercise in the luteal phase is reduced, as the sweat rate does not match the increase in rectal temperature (Pirvanik et al. 1992). The increased exercising temperature in these studies was associated with a higher heart rate of around 10 beats per minute and thus greater cardiovascular effort and often perceived exertion (Hessemer et al. 1985; Pirvanik et al. 1992). The thermoregulatory differences during exercise between the follicular and luteal phase are a consequence of elevated

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progesterone and not oestrogen, as oestrogen supplementation during the early follicular phase did not have any effect on the thermoregulatory response during moderate intensity exercise when compared to early follicular phase without supplements (Chang et al. 1998).

Theories have been proposed of a critical core temperature limiting exercise performance. Thus many athletes have used various strategies of pre-cooling to reduce their core temperature before exercise in order to delay the time to reach the critical core temperature during exercise and so possibly promote a better performance (for example, Duffield et al. 2003). Therefore the higher basal body temperature in the luteal phase should have a negative affect on performance by decreasing the time to reach critical temperature, particularly in hot conditions.

As will be discussed in the subsequent sections, few studies have reported differences in exercise performance under normal ambient conditions at high or moderate intensities between menstrual phases and of these few, most report improvements in the luteal phase. Therefore a change in the thermoregulatory set point that may persist during exercise in the luteal phase does not appear to hamper exercise performance.

1.2.2.3 Heart rate

Although Hessemer et al. (1985) and Pirvanik et al. (1992) reported significantly higher heart rate during exercise in the luteal phase coincident with the higher body temperaturein this phase, most studies have found that submaximal and maximal heart rate during exercise do not change between menstrual phases (Bemben et al. 1995; Dean et al. 2003; De Souza et al. 1990; Galliven et al. 1997; Hackney et al. 1991; Horton et al. 2002; Jurkowski et al. 1981; Nicklas et al. 1989). Some of the studies that report no change in heart rate between menstrual phases included the LF phase, in which oestrogen is elevated without progesterone, in the comparison. However, Carter et al. (2001a) found that oestrogen supplementation in men decreased heart rate during exercise at 60%VO_2max compared to the placebo condition. Conversely, Seebauer et al. (2002) recorded resting heart rate for 60 min every day over the course of two menstrual cycles and found heart rate to vary during the menstrual cycle with a minimum reading in the early follicular and a maximum in the late luteal phase, corresponding to roughly

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the last week of the cycle. The ML phase resting heart rate was significantly higher than all of the average readings during the follicular phases (menses, EF, MF, LF) but not different to that at ovulation. The heart rate at ovulation was also higher than during the follicular phases. These results correspond with similar changes recorded for basal body temperature in the study of Seebauer et al. (2002).

The relationship between heart rate and body temperature is that for every 1°C increase in temperature, heart rate increases by 7 beat per minute, which may be partially due to a direct temperature effect on the sinoatrial node (Janse de Jonge 2003). However, the increase in heart rate with rising body temperature is also a response to a drop in central venous pressure with the redistribution of blood flow to the skin in order to dissipate heat gain, and hence heart rate increases to maintain cardiac output. In this regard increases in oestrogen alone has been shown to have no effect on forearm blood flow during exercise (Chang et al. 1998). However, differences may occur in cutaneous vasodilation during exercise between the FP and LP (Hessemer et al. 1985).

Changes in plasma volume, which has been reported to occur across the menstrual cycle will also affect heart rate due to a possible change in stroke volume (Janse de Jonge 2003). However, Jurkowski et al. (1981) reported that stoke volume and cardiac output during exercise was not different between menstrual phases during exercise.

1.2.2.4 Metabolic rate

An increased basal metabolic rate post ovulation is a well-established finding (Hessemer et al. 1985; Melanson et al. 1996; Matsuo et al. 1999). In fact, the luteal phase is often associated with increases in 24-hour energy expenditure and therefore increases in energy intake to meet the additional energy requirements when compared to the follicular phase (Buffenstein et al. 1995). Although increases in metabolic rate in the luteal phase were thought to possibly be a progesterone-associated response, the increase in energy expenditure occurs faster than the increase in progesterone post ovulation (Buffenstein et al. 1995). An observed increase in free triiodothyronine (T3) (active form of thyroid hormone) in the luteal phase compared to follicular phase is most likely a contributor to the increase in metabolic rate in the luteal phase (Lariviere et al. 1994).

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In one study, a significant 5% increase in exercising energy expenditure was found in the ML phase versus the EF phase, when the exercise intensity was approximately 70% VO_2max (Hessemer et al. 1985). A further study found exercising energy expenditure to be on average 3% greater in the ML than EF phase when exercising at 80% VO2max but no difference was observed at 55% VO2max (Williams and Karenbuhl 1997). However, other studies have found that the increased resting energy expenditure in the luteal phase does not always continue to be elevated above follicular phase levels during exercise (Hackney et al. 1991; Horton et al. 2002; Jurkowski et al. 1981; Matsuo et al. 1999). An increase in metabolic rate during exercise will imply an increase in metabolic demand and cardiac effort and may also contribute to the increased body temperature in the luteal phase and hence may have implications for exercise performance.

1.2.2.5 Oestrogen's membrane stabilising and anti-oxidant properties

Oestrogen improves membrane stability and protects phospholipids from oxidative damage (Kendall and Eston 2002; Tiidus 1995, 2000). These properties reduce membrane damage caused from unaccustomed exercise and hence preserve membrane function (Kendall and Eston 2002). Such benefits could be viewed as a promoter for faster recovery between strenuous exercise sessions. These oestrogen effects where observed in male rats on oestrogen supplements and ovarectomised female rats receiving oestrogen treatment, as an attenuated post exercise creatine kinase (CK) activity response (Amelink and Bär 1986; Bär 1988; Koot 1991). However, post exercise CK levels do not correlate with the extent of histological muscle damage (Bär and Amelink 1997; Goodman et al. 1997; van der Meulen et al. 1991) and do not follow the recovery of muscle function following unaccustomed exercise (Warren et al. 1999). Therefore although increased serum concentration of CK is generally regarded as an indicator of muscle damage (Goodman et al. 1997; Janssen et al. 1989), it should probably not be used to quantify muscle damage. Rather CK efflux reflects disruption to the sarcolemma (due to mechanical stress and free radical activity) and hence increased membrane permeability (Amelink and Bär 1986; Armstrong et al. 1991; Bär and Amelink 1997; Goodman et al. 1997; Tiidus 2000). In addition CK activity returning to basal level does not necessarily mean that the muscle has fully recovered; it only indicates cessation of CK leakage from muscle and therefore restoration of membrane

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function (Janssen et al. 1989). Therefore circulating CK activity is an indicator of membrane function. However, the greater the degree of compromise to membrane function due to the action of free radicals, elevated metabolism or mechanical stress, the greater the degree of secondary damage due to calcium overload and inflammatory response (Armstrong et al. 1991; Kendall and Eston 2002) . Therefore the use of CK as a marker of membrane function in gender and menstrual phase comparative studies is appropriate as the benefits of oestrogen in the prevention of muscle damage have been targeted to mainly the preservation of membrane function (Kendall and Eston 2002; Tiidus 1995, 2000).

A gender difference in pre- and post exercise CK activity is well documented, with women producing a lesser CK response and displaying a faster recovery time from a given bout of exercise (Bär 1988; Janssen 1989; Norton et al. 1985; Shumate et al. 1979; Sorichter et al. 2001; Stupka et al. 2001). This noticeable gender difference in CK activity cannot be simply accounted for by differences in body mass, body composition (Norton 1985), muscle mass involved in exercise (Nosaka 1992) or clearance rate (Van der Meulen et al. 1991) and is therefore largely assumed to be related to the higher oestrogen concentration in women. Furthermore, eumenorrhoeic and pregnant women have lower resting CK levels than premenarchial and postmenopausal women (Arnett 2000; Smith 1979). A novel study in our laboratory investigated the influence of varying menstrual phase (EF/LF/ML) on the CK response to 20 min of downhill running and compared this to the response in a male group (Figure 5) (Herr and Oosthuyse, unpublished observation). In this study we included three separate female groups, one for each menstrual phase, as an initial bout of eccentric exercise induces adaptations for a repeated bout of eccentric exercise (Kuipers 1994). The CK response to downhill running was similar between menstrual phases. CK peaked at 24 hours post exercise and values returned to pre-exercise levels by 48 hours post exercise. Although no differences were observed for different phases of the cycle, the previously reported gender difference was observed. CK activity was higher at rest in the men and while a similar peak in CK activity was noted 24 hours post exercise in men and women, these values only returned to pre-exercise values 72 hours following the exercise bout in the men while the women had recovered already after 48 hours.

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During the EF phase of the menstrual cycle the oestrogen concentration was similar to that of the male group. Therefore we could have expected the females participating in the EF group to have a similar CK response to the exercise session as the males. Instead, the EF phase group responded similarly to the LF and ML phase groups who participated with oestrogen levels that where six and three fold higher respectively than the EF or male group. This may be due to the apparent protection that previous exposure to elevated oestrogen can confer towards membrane protection for a given period (Amelink and Bär 1986). Therefore, the female subjects in the EF group who participated with low oestrogen levels would have been protected from excessive membrane damage due to the previously high oestrogen levels that they experienced in their ML and LF phases.

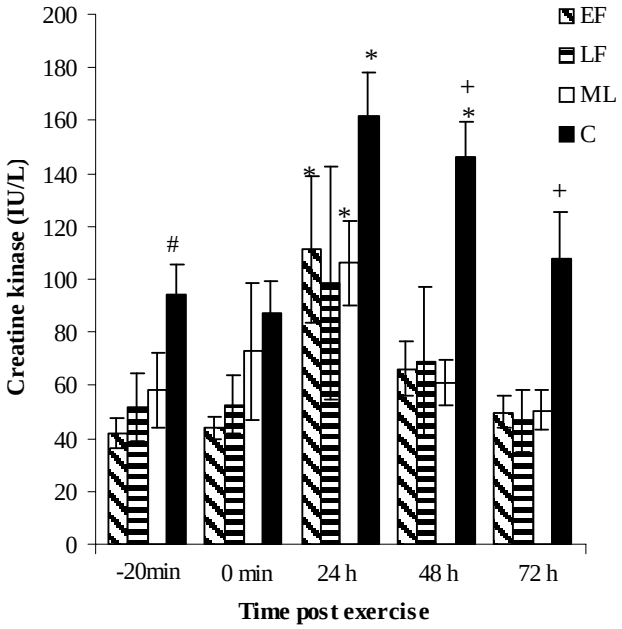

Figure 5. Serum creatine kinase (CK) activity at rest and at various time points following 20 min of downhill running at 9 km/h with a –10% gradient in a control (C) male group (n=6) and three groups of eumenorrhoeic women that participated in one of three menstrual phases (EF: Early follicular (n=6), LF: late follicular (n=5), or ML: mid-luteal phase (n=5)). # $P<0.01$, significantly greater than the combined female average for pre-exercise CK; * $p<0.05$, significantly greater than pre-exercise CK within a given group; + $p<0.05$, significantly greater than all other groups at the same time point post exercise. (Note that the 24 h value for the late follicular group did not attain significance relative to pre-exercise value ($p>0.05$) possibly due to the large inter-subject variability and small sample size. However, the 24 h value was the highest value attained post exercise for each subject within this group.)

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1.2.3 Summary of the non-reproductive physiological actions of oestrogen and progesterone and their potential consequences for exercise performance

Overall it would appear that progesterone in the luteal phase should compromise exercise performance due to the thermoregulatory, ventilatory and possibly added cardiovascular strain that should, at the very least, elevate perceived exertion. Furthermore, the many anti-oestrogen effects of progesterone that ameliorates the positive influence of oestrogen on metabolic responses to exercise would also suggest a worse exercise performance during the luteal phase. On the other hand, the literature does contain some reports of better performances in the luteal phase and is most likely a consequence of a high oestrogen to progesterone ratio that would allow oestrogen to mitigate the effects of progesterone and promote performance by inducing a favourable metabolic response. Thus the late follicular phase, although only transitory (roughly 2 days), but which coincides with the pre-ovulatory surge in oestrogen, should therefore be the optimal time for a best performance, particularly in endurance events.

1.2.4 Effects of the ovarian hormones on exercise performance

1.2.4.1 At maximal exercise intensities

1.2.4.1.1 VO_2max

Maximum aerobic capacity (VO_2max) is often used as an indication of endurance training status, athletic potential and as a predictor of performance in endurance or high intensity aerobic events. It is conceivable that VO_2max could fluctuate across the menstrual cycle as a result of possible changes to cardiorespriatory responses during exercise in various menstrual phases. However, most studies report no change in VO_2max or VO_2peak between menstrual phases (Beidleman 1999; Bemben et al. 1995; Casazza et al. 2002; De Souza et al. 1990; Dombovy et al. 1987; Schoene et al. 1981) besides Lebrun et al. (1995) who observed an average 2% higher VO_2max in the EF versus the ML phase that was significant when expressed as L/min ($p=0.04$) and almost significant when expressed in ml/kg.min ($p=0.06$). Moreover, a recent study has shown that the use of oral contraceptives decreases VO_2peak by 13% compared to a naturally cycling condition or eumenorrhoea (Casazza et al. 2002). The decrease in VO_2peak

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corresponded with a decrease in peak power output and time to exhaustion as well as an increase in body mass and percentage body fat, despite a similar dietary intake (Casazza et al. 2002). However, these authors speculated that the drop in VO_2peak with oral contraceptive use was due to an attenuation of sympathetic nerve activity that results in a decrease in vasoconstriction and hence a lower mean arterial pressure. Another explanation is that since less work was done in the VO_2peak test, oxygen requirement was reduced and this was reflected in a lower VO_2peak.

1.2.4.1.3 Lactate Threshold and ventilatory anaerobic threshold

A change in the lactate or "anaerobic" threshold could alter substrate selection at a particular percentage of VO_2max or high intensity exercise performance. However, Dean et al. (2003) have reported no significant change in the lactate threshold between EF, MF and ML phases. In addition, others have reported no difference in the onset of the anaerobic ventilatory threshold between menstrual phases (Dombovy et al. 1987; Schoene et al. 1981). On the other hand, Bemben et al. (1995) found that the ventilatoy threshold occurred at a higher percentage of VO_2max in the EF phase compared to the LF and ML phase, but there was no change in time to exhaustion during a high intensity incremental treadmill run.

1.2.4.1.4 Short duration high intensity time to exhaustion

Short duration high intensity exercise places a huge demand on glycolytic and aerobic carbohydrate utilisation pathways and the cardiorespiratory system. All studies to date except one, however, have not observed any influence of the menstrual cycle on time to exhaustion during such high intensity bouts of exercise (Bemben et al. 1995, Dean et al. 2003; De Souza et al. 1990; Dombovy et al. 1987; Jurkowski et al. 1981; Lebrun et al. 1995). The exception is the study of Schoene et al. (1981) who reported a worse performance in the mid-luteal phase compared to early follicular phase, but only in their non athletic (or sedentary) group, while they too, found no difference in performance in the well-trained athletes. Nonetheless, these authors claim that the impaired performance in the luteal phase in the non-athletes was a result of an increased feeling of dyspnea associated with the elevated ventilatory rate during exercise in this menstrual

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phase, while training mitigated the impact of the increased ventilatory rate on performance (Schoene et al. 1981).

1.2.4.2 Submaximal exercise intensities

1.2.4.2.1 Time to exhaustion

Ovarectomised rats on oestrogen treatment improved time to exhaustion in a prolonged submaximal incremental treadmill run by 20% when treated with a very low oestrogen dose (0.02 µg/100g) when compared to sham injected rats (Kendricks et al. 1987). Endurance time continued to increase with increasing oestrogen dosing (increases in oestrogen up to 0.1 µg/100g) resulting in up to a 42% improvement over sham-injected controls (Kendricks et al. 1987). Further increases in oestrogen dosing at physiological levels did not increase endurance time over the 42% improvement level (Kendricks et al. 1987). However, a pharmacological dose of 10 µg/100g resulted in a 50% improvement in time to exhaustion compared to the sham injected animals (Kendricks et al. 1987). These massive improvements to endurance capacity coincided with glycogen sparing in the red and white vastus muscle, myocardium and liver (Kendricks et al. 1987).

Similar results have been shown in human studies. Two studies have reported an effect of menstrual phase on endurance capacity. The first study found that following 40 min of submaximal incremental cycling, time to exhaustion at 90% of maximum power output was doubled in the ML phase compared to the MF phase (Jurkowski et al. 1981). This coincided with lower blood lactate levels in the ML phase. The second study had a smaller sample size (n=6) and therefore the (on average) 10% longer time to exhaustion at 70% VO_2max in the ML phase compared to the MF phase did not quite reach significance ($p<0.07$) (Nicklas et al. 1989). Conversely, a further study (n=8) that also compared time to exhaustion at 70% VO_2max did not find any difference between the EF and ML phase (Beidlemen et al. 1999). Furthermore, Bailey et al. (2000) also compared time to exhaustion at 70%VO_2peak between the EF and ML phase on two occasions, once with and once without carbohydrate supplementation during exercise. No menstrual phase effect was observed with or without carbohydrate supplementation. While all of these studies demonstrated an increase in oestrogen from the follicular to

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luteal phase of greater than or equal to 2-fold, the calculated oestrogen to progesterone ratio differed noticeably such that the studies reporting a better performance in the luteal phase had higher E/P ratio, while the studies that found no change in endurance time had a lower E/P ratio (Table 2). This observation implies that the higher relative progesterone concentration in the latter studies impeded the metabolic benefits of oestrogen that may have been more prominent during the LP of the former studies.

It should be borne in mind when considering these studies that time to exhaustion at submaximal exercise intensity is a measure of endurance capacity and is not a direct measure of exercise performance (Jeukendrup et al. 1996), although it can provide an indication of an athlete's potential for endurance events. Such protocols do not have a high reproducibility and studies have reported coefficients of variation as high as 30% when using these tests (Jeukendrup et al. 1996) reducing the statistical power of comparison between interventions, in this case between menstrual phases.

Table 2. Relative changes in the ovarian hormones between the follicular and luteal phase in relation to submaximal endurance performance

Reference	Magnitude of increase in oestrogen in LP above FP	Oestrogen to progesterone ratio in LP	Result
Time to exhaustion at submaximal intensity			
Bailey et al. 2000	2.28-fold	12.3	NS; EF vs. ML
Beidlemen et al. 1999	2.87-fold	8	NS; EF vs. ML
Jurkowski et al. 1981	2-fold	21.3	P<0.02; ML>MF
Nicklas et al. 1989	3.85-fold	18	P<0.07; ML>MF
Time trial performance			
Campbell et al. 2001	2.3-fold	5.5	P<0.05 MF faster than ML without CHO supplement
Campbell et al. 2001	2.5-fold	6	NS; MF vs. ML with CHO supplement

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1.2.4.2.2 Time trial performance

Exercise protocols with a fixed end-point, such as time to complete a given distance, or to expend a given amount of energy or distance covered in a fixed time period etc. are a good measure of exercise performance, having high test-retest reproducibility as described by a low coefficient of variability (1-3%) (Bishop 1997; Hickey et al. 1992; Jeukendrup et al. 1996; Palmer et al. 1996). Only one study has measured time trial performance between menstrual phases (Campbell et al. 2001). This study compared the time to expend a given amount of energy after completing a 2 h submaximal session at 70%VO_2max in the MF and ML phase with and without carbohydrate supplements in overnight fasted subjects. They observed a 13% improvement in time trial performance in the MF phase without carbohydrate supplementation during exercise, which was associated with higher carbohydrate use and whole body rate of glucose appearance (hepatic glucose production) and rate of disappearance (or glucose uptake) suggesting a better capacity for carbohydrate use in the MF phase. An increased capacity for carbohydrate utilisation is beneficial in short duration time trial events which take place at high intensities. This observation of better time trial in the MF phase (Campbell et al. 2001) coincides well with another study from the same authors who found oestrogen to promote contraction stimulated glucose uptake and hepatic glycogenolysis during exercise in ovarectomised rats, while progesterone antagonised these responses (Campbell and Febbraio 2002). The pre-exercise MF phase average oestrogen concentration was relatively high (360 pmol/L) (Campbell et al. 2001) and hence oestrogen may have promoted glucose use during these trials. In addition despite a 2.5-fold increase in oestrogen in the ML phase over the MF phase, the oestrogen to progesterone ratio in the ML phase was comparatively low (E/P of 5-6) (Campbell et al. 2001) (Table 2). Thus the relatively high progesterone concentration during these trials countered the benefits of an elevated oestrogen concentration and produced a worse performance. However, the use of carbohydrate supplements during exercise elevated glucose rate of appearance, disappearance and plasma glucose use, providing sufficient fuel-of-choice to promote an optimal performance in a short duration high intensity time trial, regardless of menstrual phase (Campbell et al. 2001). It may be interesting to compare time trial performance over a longer event between menstrual phases with a higher oestrogen to progesterone ratio in the luteal phase or when oestrogen alone is

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elevated such as during the pre-ovulatory surge in oestrogen. Therefore, the last research study presented in this thesis (Chapter 6) assessed cycling time trial performance during the early follicular, late follicular and mid-luteal phase of the menstrual cycle. The inclusion of the LF phase in this comparison is a novel contribution to the literature and is motivated by the many oestrogen-induced metabolic effects already discussed that should promote performance in such an event.

Part 3

1.3 Stable tracer methodology to estimate plasma free fatty acid oxidation rate and kinetics

1.3.1 Estimating plasma FFA kinetics

1.3.1.1 General concepts

The adipose tissue contains the largest proportion of the body's fat stores with only very small amounts being stored by other tissues, including muscle (Jeukendrup et al. 1998). In addition, the body's capacity for de novo FFA synthesis is small and considered negligible (Hellerstein et al. 1989). Therefore FFAs are released into circulation from hydrolysis of triacylglycerols primarily from adipose stores or directly from dietary intake and are taken up by the liver, oxidative skeletal muscle fibres and the myocardium. The triacylglycerol stores in the adipocytes are in a cycle of continuous turnover and is referred to as the triacylglycerol-free fatty acid cycle (TG-FFA cycle) where triacylglycerols are hydrolysed and reesterified and the relative activity of lipolysis to that of reesterification determines the proportional release or appearance of FFA into circulation (Wolfe 1992b). Although this system has been classified as one of the "futile cycles", this process allows the adipocytes to respond rapidly to changes in FFA demand (Leibel and Hirsch 1985). Lipolytic rate is generally regulated by the sympathetic nervous system, circulating epinephrine, insulin and lactate concentration, whereas reesterification is dependent on the rate of rephosphorylation of glycerol (mainly) in the liver; which is dependent on the availability of the glycolytic intermediate dihydroxyacetone phosphate and hence glycolytic rate (Jeukendrup et al. 1998). FFA release from adipose stores is also dependent on the transport capacity of

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the blood. Ninety nine point nine percent of fatty acids in plasma are transported bound to albumin and the remaining 0.1% is unbound and it is this small fraction that determines the rate of FFA uptake into tissues (Jeukendrup et al. 1998). The uptake of long chain fatty acids across the sarcolemma into muscle fibres is carrier-mediated and hence follows saturation kinetics (Jeukendrup et al. 1998).

FFA release and uptake can be estimated by a constant infusion-isotope dilution technique (Wolfe 1992). Labelled palmitate or oleate are the long chain fatty acids normally chosen as the tracer for estimating FFA kinetics as they are typical of other fatty acids and reflect whole body FFA kinetics. Due to the hydrophobic nature of FFAs the tracer must be complexed to a carrier to hold the tracer in solution for the purpose of intravenous infusion. Human serum albumin, being the natural endogenous carrier for FFA in plasma, is routinely used as a carrier for the FFA tracer in the infusate. However, albumin is the main plasma protein contributing towards colloid oncotic pressure and therefore the amount of exogenous albumin administered to healthy subjects must be carefully controlled. Furthermore, human serum albumin is a blood product and although stringently extracted and sterilised of possible blood impurities, carries a negative stigma and is not without some degree of risk. In Chapter 4, we consider the use of an alternative carrier for FFA tracer infusion studies.

The intravenous infusion rate of the FFA tracer is determined by estimating the maximal endogenous FFA rate of appearance (Ra_{est}) and considering the sensitivity of the equipment used for measuring isotopic enrichment (Jeukendrup, personal communication). Therefore the infusion rate is calculated by Ra_{est} multiplied by the lowest percentage enrichment detectable. Thus, as sensitivity is normally around 2%, the proportional amount of tracer infused relative to endogenous FFA Ra, is very small. Then by means of a constant infusion of palmitate tracer the actual rate of endogenous unlabelled palmitate (or tracee) appearance is calculated based on the dilution of the infused tracer (Wolfe 1992) (see section 1.3.1.3 for equations).

1.3.1.2 Infusion and sampling site

The accuracy of the isotope dilution technique is dependent on the following assumptions: the volume of fatty acid distribution should remain constant; the pool

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should remain well-mixed so that the new fatty acids entering mix quickly with others already present; and there must be no discrimination between fatty acid tracers and tracees (Wolfe 1992).

Observations of the washout period following a bolus dose of tracer demonstrates that FFA distribution fits a 2 compartment-model as the rate of decay of enrichment does not follow a single exponential curve but rather a dual exponential function (Wolfe 1992). However, the two pools have a similar rate parameter and isotopic equilibrium is achieved within 20 min in the first pool and 15 to 20 min later in the second pool, therefore requiring approximately 40 min in total to achieve isotopic steady state. Therefore, since the kinetics of the first and second pools are the same and the second is dependent on the first, overall Ra/Rd can be based on a single pool model. In a single pool model the infusion and sampling sites do not matter (Wolfe 1992). However, with FFA kinetics it is important to use a venous-arterial sampling mode to avoid over-dilution of the tracer by concomitant tracer uptake by metabolic tissue and release of endogenous FFA by lipolysis that is measured with mixed-venous sampling, as sampling occurs before bypassing the infusion site (Figure 6). Thus in mixed-venous sampling the enrichment at which tissue uptake occurred is underestimated due to dilution of the tracer by lipolytic release of tracee, that in the case of arterial sampling would be setoff with the appearance of tracer, as the blood will pass the infusion point before a sample is drawn (Wolfe 1992). The over-dilution with mixed venous sampling can lead to overestimation of FFA Ra by approximately 21% (Jensen et al. 1988). Sampling arterialised-venous blood by using the heated hand-vein technique has been validated as an acceptable means of obtaining arterial samples (Jensen and Heiling 1991). Furthermore, no bolus-priming dose is necessary prior to commencing the constant tracer infusion as the pool size is small and the turn over rate is rapid (Wolfe 1992). The total whole body FFA Ra can be estimated by dividing the calculated palmitate Ra by the proportional contribution of palmitate to total plasma FFA concentration (Wolfe 1992).

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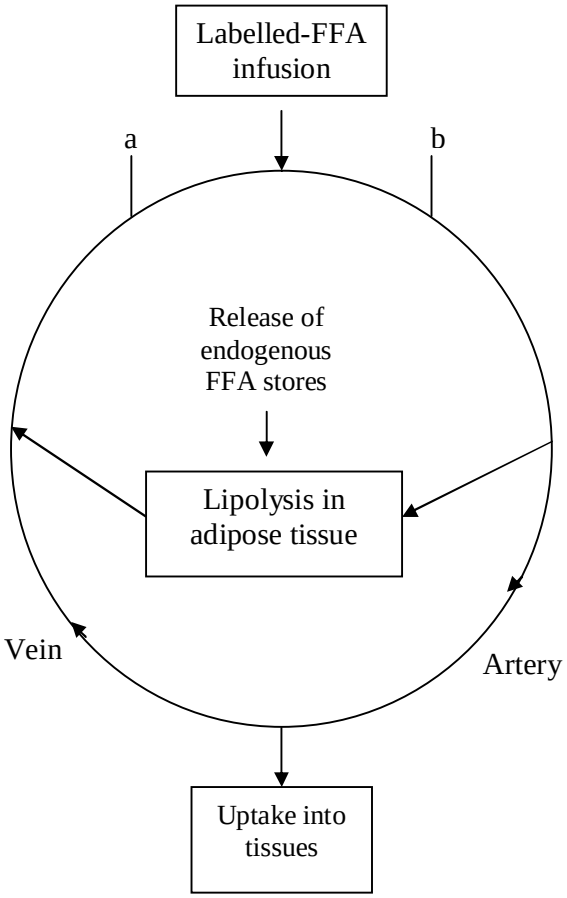

Figure 6. Adapted from Wolfe 1992; figure 9.1. Necessity of the venous-arterial sampling model for constant infusion tracer estimates of FFA Ra is evident as venous sampling from point "a" results in over dilution of the tracer due to tracer FFA uptake and lipolytic release of tracee FFA. Sampling from arterial site "b" is post the site of exogenous tracer Ra (infusion) that neutralises the effect of lipolysis.

1.3.1.3 Equations for estimating substrate kinetics based on isotope dilution

At steady–state (i.e. constant isotopic enrichment), the rate at which the tracer is infused maintains a consistent proportion of tracer to tracee in the pool of distribution in relation to the endogenous production or rate of appearance, and hence the rate of endogenous appearance of the FFA tracee can be calculated based on infusion rate (F) and plasma enrichment (E) (Wolfe 1992).

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$Ra = F/E$

Under steady-state conditions, Ra=Rd. However, under non steady-state conditions, for example during exercise, substrate kinetics can be described by equations derived by Steele et al. (1959) where changing substrate (tracee) concentration (C) and enrichment are considered and allowance is made for the fraction of the pool's volume of distribution that is rapidly mixing, i.e. the effective volume of distribution (pV) (Wolfe 1992).

$$Ra\ (\mu mol/kg.min) = \{F - pV[(C_2-C_1)/2] \times [(E_2-E_1)/(t_2-t_1)]\}/[(E_2+E_1)/2]$$

$$Rd\ (\mu mol/kg.min) = Ra - pV(C_2-C_1)/(t_2-t_1)$$

Where C_1 and C_2 is the substrate (tracee) concentration at sampling time t_1 and t_2 and E_1 and E_2 is the enrichment of the tracer in plasma at sampling time t_1 and t_2. Various values for pV have been used with 40 ml/kg being the most frequent choice, 60 ml/kg has also been used, while Jensen et al. (1990) suggests that 90 ml/kg (1.8 x plasma volume) is the most suitable value for increasing or decreasing, but particularly with decreasing, FFA inflow such as with rising insulin concentration. In addition, when stable tracers are used, their contribution to Ra cannot be considered "weightless" and therefore the infusion rate must be subtracted from the calculated Ra.

Once Rd has been calculated it is possible to calculate the metabolic clearance rate (MCR) as:

$$MCR\ (ml/kg.min) = Rd/C$$

MCR accounts for the influence of substrate concentration on the rate of uptake and in essence is the amount of plasma completely cleared of the tracee in 1 minute (Wolfe 1992).

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1.3.2 Estimating the rate of plasma FFA oxidation

1.3.2.1 General concepts

Most of the FFA taken up by muscle cells are oxidised in the mitochondria for energy production, while small amounts are oxidised in peroxisomes, or are used for replenishing the intracellular cytoplasmic pools or for synthesis of phospholipids that constitute the cell membranes (Van der Vusse and Reneman 1996). As adipose tissue is capable of storing large quantities of fat (far more than the body is capable of storing carbohydrate) it is an important energy source and total fat oxidation contributes 50% of the energy demands at rest (Jeukendrup et al. 1998). The main source of oxidisable fat is therefore plasma FFA derived from adipose stores. However, intracellular cytoplasmic lipid vacuoles, fat droplets stored between muscle fibres and triacylglycerols transported in the blood as very low density lipoproteins may also be secondary, but possibly less important sources of oxidisable FFA.

It is common knowledge that fat oxidation is imperative for sustaining exercise over long periods; Jeukendrup et al. (1998) makes the deduction that endurance athletes would only be capable of a maximum of 90 min of exercise relying solely on endogenous carbohydrate stores. While FFA oxidation supplies more energy per gram than carbohydrate, it utilises more oxygen and functions at a slower rate, thus explaining why carbohydrate becomes the major energy source at higher exercise intensities (Jeukendrup et al. 1998). FFA oxidation is therefore important at all submaximal intensities below 80%VO_2max (Romijn et al. 1993) and in fact contributes proportionally more energy than carbohydrate until the crossover point as described by Brooks et al. (1994) that occurs roughly at 60%VO_2max. In addition, maximum fat oxidation occurs between 55 and 70% VO_2max in endurance-trained men (Jeukendrup et al. 2003) but is dependent on the type of exercise (running or cycling) and gender variations regarding the intensity that produces the maximum rate of fat oxidation has been reported (Knechtle et al. 2004). However, FFA oxidation is dependent on a critical amount of tricarboxylic acid cycle intermediates (TCAI), which requires continued supply of pyruvate and hence the continued maintenance of a carbohydrate source is essential for optimal FFA oxidation rates (Jeukendrup et al. 1998). Thus exercise

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intensity is forced to drop below 50%VO_2max once carbohydrate stores are depleted due to a shortage of TCAI (Jeukendrup et al. 1998). Although plasma FFA availability and cellular uptake are also important determinants of plasma FFA oxidation, they do not appear to be the main or only limiting, variables (Jeukendrup et al. 1998; Roepstorff et al. 2004). Other limiting variables include: transport of LCFA into mitochondria is an important regulatory site (thoroughly reviewed by Kiens 2006) as well as the activity of the enzymes that initiate reesterification and so regulate the proportion of FFA shunted toward beta-oxidation or triacylglycerol synthesis. In addition the activity and amount of beta oxidative enzymes will also determine the rate of FFA oxidation.

Some studies employing metabolic tracers have assumed that 100% of FFA Rd approximates the rate of plasma FFA oxidation during exercise (Romijn et al. 1993). However, actual measures of plasma FFA oxidation have found that only 50 to 75% of FFA Rd is oxidised during moderate exercise (Friedlander et al. 1998; Friedlander et al 1999; Martin et al. 1993); albeit one study that did report close to 100% of FFa Rd oxidised in women during moderate exercise (Roepstorff et al. 2002). Therefore, plasma FFA oxidation rate should be directly measured from the recovery of labelled carbon dioxide in breath during a continuous infusion of a carbon-labelled fatty acid. Either a FFA tracer with the carbon labelled in position 1 or FFA tracer with uniformly labelled carbons is routinely used as stable or radioactive tracers. The complete oxidation of a FFA tracer molecule e.g. palmitate, with the label in the carbon-1 position will result in only 1 labelled CO_2 being recovered and hence a 1:1 ratio is applicable. However, when determining molar fatty acid oxidation rate with uniformly labelled carbon tracers the recovery of labelled CO_2 must be divided by the number of carbons in the fatty acid chain.

This methodology assumes that (1) all labelled CO_2 recovered originates solely from the oxidation of the infused fatty acid tracer and, (2) all CO_2 liberated from FFA oxidation are recovered almost immediately in expired air.

1.3.2.2 Correcting for background carbon-13 recovery

In the case of carbon-13 isotopes, the first assumption is invalid as the body has its own endogenous store of carbon-13 derived from a mixed diet. The C-4 photosynthetic

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pathway (or dicarboxylic acid pathway) naturally produces more carbon-13 than the more common C-3 (or Calvin) photosynthetic pathway and hence carbon-13 is abundantly present in various cane and maize-rich starches (Lacroix et al. 1973). Thus carbon-13 has a higher abundance in carbohydrate and protein stores than in lipid stores (Lacroix et al. 1973). Therefore, account must be made for the proportion of carbon-13 labelled carbon dioxide arising as a result of the oxidation of endogenous stores to prevent over-estimation of the fraction arising from the oxidation of labelled tracer. The enrichment of background expired air samples that are taken before commencing infusion of the tracer are subtracted from the enrichment measured in samples taken during the infusion time. However, this straightforward correction is complicated by a change in metabolic state that is associated with a change in the relative contribution of carbohydrate and fat to energy expenditure such as during exercise (Wolfe et al. 1984). An increase in carbohydrate oxidation corresponds with an increase in endogenously derived labelled carbon dioxide due to the greater presence of carbon-13 in glycogen stores. The changes from rest to exercise or changing exercise intensity, or supply of exogenous substrate are examples where background carbon-13 in expired air may vary (Wolfe et al. 1984). In the example of Wolfe et al. (1984), RER was drastically increased by exercise combined with an insulin/glucagon clamp, from 0.74 to 0.94 which resulted in an increase in endogenous carbon-13 carbon dioxide production (enrichment increased from 0.0032 to 0.0058 atom percent excess) due to a major shift from fat to carbohydrate oxidation. During submaximal exercise at a moderate intensity over a prolonged duration, the magnitude of increase in RER is less severe and will progressively drop to eventually approximate resting levels (Figure 7). Thus under such conditions it can be expected that background carbon-13 carbon dioxide should remain fairly constant. In a pilot trial we observed negligible variation in background carbon-13 enrichment in expired air during 90 min of cycle ergometry at 60%VO_2max performed 2 hours postabsorptively (Oosthuyse, unpublished observation) (Figure 7). In addition, proportionally small errors in background will be of little concern when the level of enrichment of expired carbon dioxide resulting from the isotope infusion is high (Wolfe et al. 1984).

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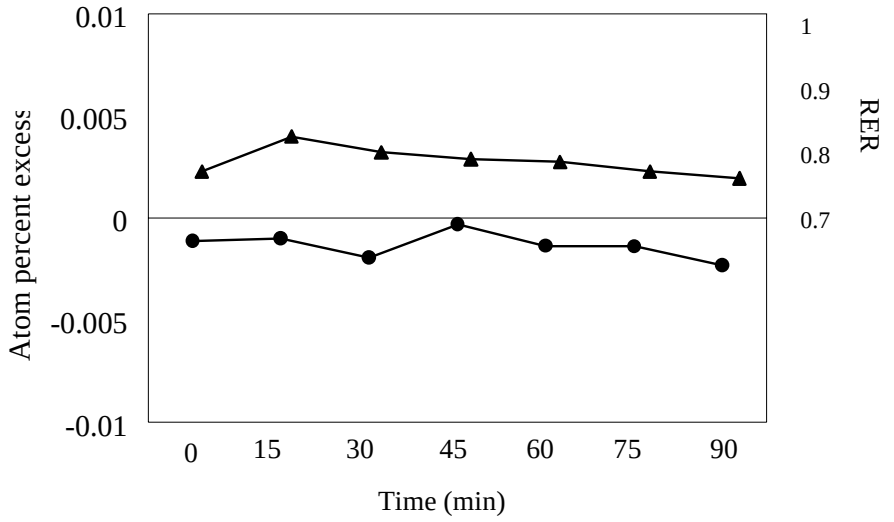

Figure 7. Background carbon-13 enrichment (●) in expired carbon dioxide samples taken before and during 90 min of cycling exercise at 60% VO_2max in a non fasted female subject with corresponding RER values (▲) at each time interval.

1.3.2.3 Correcting for delays in label recovery due to label fixation

The second assumption that all CO_2 liberated from the oxidation of a labelled tracer are recovered almost immediately in expired air is not correct, as a proportion of liberated CO_2 may be trapped in the bicarbonate buffering system or in secondary products of the tricarboxylic acid cycle (Sidossis et al. 1995). The bicarbonate buffering system functions as a means of transporting CO_2 from tissues into blood and to the lungs and so maintains blood pH. It also buffers the proton release from lactic acid production. At rest the delay in CO_2 recovery due to bicarbonate kinetics is significant (recovery is approx. 77%). However, this system is accelerated with increases in metabolic rate such as during exercise. The resultant recovery of CO_2 from bicarbonate is greater than 90% during exercise (Sidossis et al. 1995a; Trimmer et al. 2001). Nonetheless, most carbon tracer studies administer a bicarbonate prime to bring about rapid equilibrium within the blood bicarbonate pool. However, Heiling et al. (1991) observed that bicarbonate kinetics could not completely explain the delay in CO_2 recovery from the uptake and oxidation of a labelled FFA tracer and challenged the validity of isotopic measures of plasma FFA oxidation. Subsequently, Sidossis et al. (1995b) proved that the carbon

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label in the form of acetyl CoA arising from the beta oxidation of the fatty acid tracers can be channelled into the products of secondary reactions that occur with the tricarboxylic acid cycle intermediates. This accounted for the incomplete recovery of the labelled carbon following fatty acid tracer infusions as observed by Heiling et al. (1991) (Figure 8). Confirmation of this theory was provided by observation of a similar rate of decay of labelled CO_2 in expired air following the termination of a labelled actetate (immediately convert to acetyl CoA intracellularly) and palmitate infusion (average half-life of labelled CO_2 decay 107 and 97 min, respectively) (Sidossis et al. 1995b). Whereas labelled CO_2 decay following bicarbonate infusion occurs four times faster than the rate of CO_2 decay following palmitate infusion (average half life of labelled CO_2 decay 37 and 141 min, respectively) (Heiling et al. 1991). A similar response is observed during exercise only with an increase in the rate of decay of the label in all pools by several fold compared to when at rest due to an overall increase in metabolic rate (Figure 8).

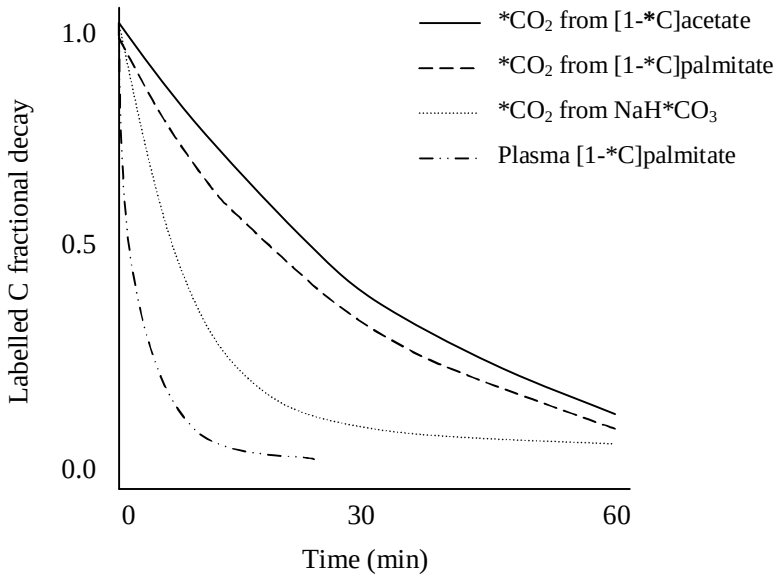

Figure 8. Diagrammatic representation of Sidossis et al. (1995b) Figure 5, presenting the rate of decay of the carbon label (*C) in plasma palmitate and in expired carbon dioxide (*CO_2) after stopping the infusion of a carbon-labelled palmitate, carbon-labelled sodium bicarbonate (NaH*CO_3) and carbon-labelled acetate tracer during exercise.

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The secondary reactions of TCAI that cause label fixation include the transamination reactions between alpha ketoglutarate and glutamate/glutamine, and oxaloacetate and aspartate; as well as the gluconeogenic reaction that involves the exchange between oxaloacetate and pyruvate and proceeds to form either lactate or glucose (Figure 9) (Sidossis et al. 1995b). These reactions are in equilibrium and therefore essentially involve the exchange of carbon skeletons and so as the labelled carbon passes through the TCA following beta-oxidation of the tracer, there is a risk that the carbon-13 may be exchanged for a carbon-12. Subsequently, a carbon-12 CO_2 is liberated in place of a carbon-13 CO_2 resulting in the oxidation of the labelled tracer being undetected (Sidossis et al. 1995b). Hence, plasma FFA oxidation rate is underestimated. However, the carbon labels that are fixed in the products of these secondary reactions are all oxidisible substrates and therefore the trapped label will eventually appear in expired CO_2. Unfortunately, the time required to establish equilibrium of the carbon label in these secondary pools (15 h) is substantially longer than the duration of most studies (Mittendorfer et al. 1998). Sidossis et al. (1995a) proposed a correction factor that will account for the delay in *CO2 recovery following FFA tracer oxidation caused by both the retention in intermediatory metabolic pathways and in the endogenous bicarbonate pool, termed the acetate correction factor.

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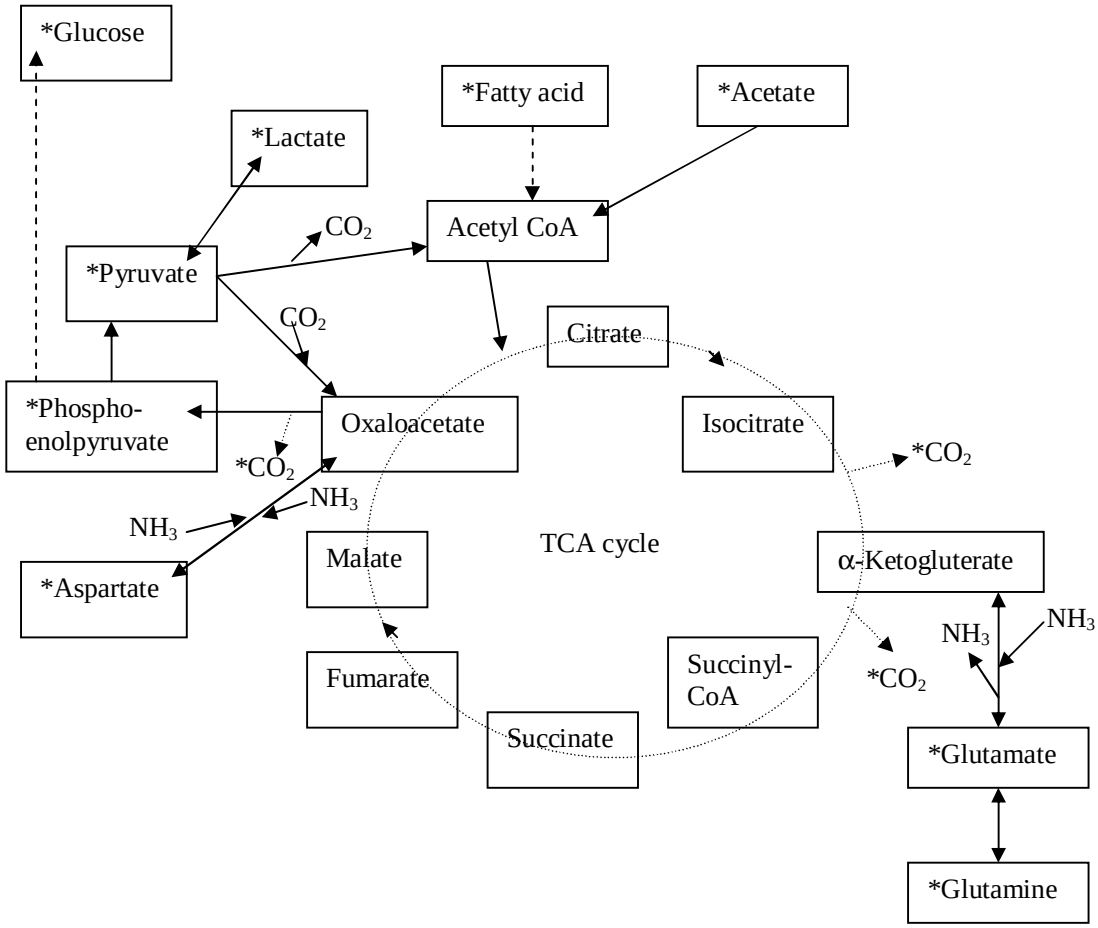

Figure 9. The main possible sites of carbon label fixation in metabolic intermediate reactions or points of recovery as carbon dioxide (CO_2) following entry of the carbon label into the tricarboxylic acid (TCA) cycle. Long chain fatty acids are completely catabolised to acetyl CoA units by repeated passage through the beta-oxidation pathway. Thus the carbon label identified as * from a fatty acid or acetate tracer enters the TCA as acetyl CoA. This figure is modified from Sidossis et al. (1995b) Figure 1. NH_3 is ammonia concurrently incorporated into the TCA cycle intermediate carbon skeleton during transamination in the production of an amino acid and released by deamination from a donor amino acid to replace the TCA cycle intermediate.

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1.3.3 The acetate correction factor

The acetate correction factor is the fractional recovery of labelled CO_2 from a constant infusion of carbon labelled acetate. The infused acetate is converted to acetyl CoA and enters the TCA cycle as if it were derived from the beta-oxidation of a FFA. Uptake of acetate from the vascular compartment occurs by simple diffusion (Mittendorfer et al. 1998), is converted by acetyl CoA synthetase to form acetyl CoA and directly enters the TCA cycle (Sidossis et al. 1995b). Thus the acetate correction factor accounts for label fixation from the point of entry into the TCA cycle to appearance of the label in expired air. The carbon tracer estimate of plasma FFA oxidation rate is corrected for underestimation due to total label retention in subsidiary reactions with TCAI and the bicarbonate pool by dividing the calculated plasma FFA oxidation rate by the value derived for the acetate correction factor. The following equations apply:

Fractional recovery of $*CO_2$ from *acetate (a_r) = (ECO_2 x VCO_2)/F

Where $*CO_2$ is labelled CO_2, *acetate is labelled acetate infusion and a_r is the acetate correction factor. ECO_2 is the enrichment of the carbon label in expired air and VCO_2 is carbon dioxide production as μmol/kg.min. F is the infusion rate in μmol/kg.min.

Plasma FFA oxidation = (ECO_2 x VCO_2)/(Ep x a_r)

Where Ep is the palmitate tracer enrichment in plasma (μmol/kg.min) measured during an experimental trial.

However, a number of variables should be considered that influence the acetate correction factor. In addition many of these variables are influenced by menstrual phase or changes in the ovarian hormones (as will be discussed in the subsequent sections) and therefore, study 1 (Chapter 2) of this thesis evaluates the effect of menstrual phase on the acetate correction factor during submaximal exercise.

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1.3.3.1 Infusion time

While isotopic equilibrium of the acetate tracer can be rapidly achieved (90 min at rest and 60 min during exercise) due to a small fatty acyl CoA pool, this only represents steady state in the primary pool (Mittendorfer et al. 1998). Equilibrium in the secondary pool that includes the products of the TCAI exchange reactions will take substantially longer, however only once this has occurred is true steady state achieved (Mittendorfer et al. 1998). Nonetheless, it is more appropriate to derive the acetate correction factor based on equilibrium of the primary pool as the carbons of acetyl CoA derived from the FFA tracer will behave similarly to the acetate tracer infused over the same time frame and during this initial phase will only provide a measure of label fixation rather than combined label fixation and secondary product oxidation (Mittendorfer et al. 1998). In addition, measurable quantities of labelled CO_2 from secondary pool oxidation, that will underestimate the extent of label fixation, will only occur after prolonged duration of infusion, and thus in fact renders long infusion periods at rest prior to exercise in attempts to ensure isotopic steady state during the exercise period, counter intuitive (Trimmer et al. 2001). Furthermore emphasis is placed on keeping the infusion time constant for both labelled acetate and FFA trials.

1.3.3.2 Position of the carbon label

Fractional recovery of labelled CO_2 in expired air due to the infusion of an acetate tracer is also dependent on the position of the labelled carbon, i.e. carbon-1 (C-1) or carbon-2 (C-2) position. When carbon-1 of the acetate tracer carries the label, fractional recovery is approximately 20% greater at rest and 17% greater during moderate intensity exercise than with the label in carbon-2 position (Trimmer et al. 2001). In general the carbons of acetyl CoA entering the TCA will be retained in TCA intermediates such that the carbons in position 1 of acetyl CoA will only be released as CO_2 on the second spin of the cycle while those in position carbon-2 are released from the third spin of the cycle (Ruzzin et al. 2003). During the second turn of the cycle 50% of labelled C-1 is released before alpha ketoglutarate and 50% after alpha ketoglutarate (Ruzzin et al. 2003). Only 50% of total label C-2 is released in the third spin of the TCA and the remaining 50% will be released progressively over a number of subsequent spins of the cycle (Ruzzin et

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al. 2003). Therefore the extra spins through the cycle for labelled carbon-2s promotes an increased chance of these carbons participating in exchange reactions and hence causing label fixation and reducing fractional recovery. For this reason the acetate tracer used for deriving the acetate correction factor must be labelled identically to the FFA tracer that will be used for determining plasma FFA oxidation rate. Therefore when using [1-*C]FFA tracer, [1-*C]acetate is appropriate for the derivation of the correction factor and likewise [U-*C] FFA requires the use of [1,2-*C]acetate.

Interestingly, as glucose catabolism also involves the TCA cycle it may be considered necessary to account for label fixation in the TCA when quantifying plasma glucose oxidation using carbon tracers (Shrauwen et al. 1998). However, glucose tracers with the label on carbon 3 and 4 are released before entry into the TCA at pyruvate decarboxylation and therefore are excluded from the risk of label fixation via TCA exchange reactions (Ruzzin et al. 2003). Carbon labels in position 1 and 6 on the glucose tracer, however, will enter TCA on carbon-2 of acetyl CoA and glucose labelled at position 2 and 5 will enter the TCA as acetyl CoA with the label on C-1 (Ruzzin et al. 2003). Nonetheless, label fixation in TCA exchange reactions is less likely to occur when acetyl CoA is derived from glucose as more CO_2 is produced per kilojoule released (Shrauwen et al. 2000) and the rate of energy production is faster when compared to FFA catabolism (Jeukendrup et al. 1998). In addition this is particularly true when large doses of exogenous glucose are administered where the flux of the carbon label through the TCA cycle far exceeds the kinetics of the TCA exchange reactions (Ruzzin et al. 2003).

1.3.3.3 Metabolic rate and metabolic state

The fractional recovery of labelled CO_2 from acetate tracer infusion (acetate correction factor) increases in relation to metabolic rate and fits a single exponential model as described by Sidossis et al. (1995a) over a range of exercise intensities (Figure 10).

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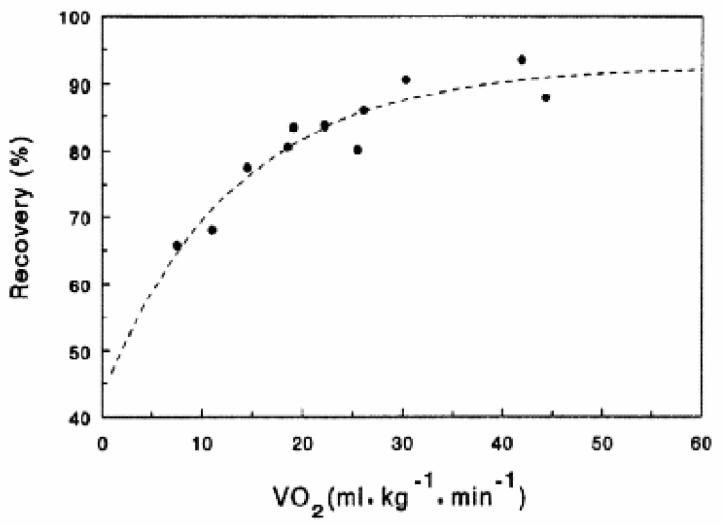

Figure 10. Relationship between the acetate correction factor (measured as % recovery of the carbon label in expired air from an acetate tracer infusion) and energy expenditure during exercise as described by Sidossis et al. (1995a). This relationship fits a single exponential model: acetate carbon recovery (%) = 92.68 x (1 – 0.5338 x $e^{-0.00753 \times VO2}$). This figure has been reproduced from Sidossis et al. (1995a) Figure 1.

Schrauwen et al. (2000) have shown that absolute VO_2 expressed relative to fat free mass is a major variable determining variability in the acetate correction factor between subjects at rest and during exercise. The rate of TCA cycling increases with increases in energy demand without a corresponding increase in the rate of TCAI exchange reactions, thereby limiting the chance of label fixation in these pools and increasing the chance for direct appearance in VCO_2. While the inter-individual variation in the correction factor (coefficient of variation 12-16%) appears to be relatively high (Schrauwen et al. 2000), the reproducibility within a subject is good (coefficient of variation, 4%) (Schrauwen et al. 1998). Nonetheless, if the derivation of the acetate correction factor and the measurement of plasma FFA oxidation cannot be conducted simultaneously and therefore takes place on separate occasions, factors altering metabolic rate/state need to be considered. In this respect, when including women it may be necessary to control for menstrual phase specifically with measurements conducted at rest as a variation in basal metabolic rate across the menstrual cycle is well documented (Matsuo et al. 1999). While most studies have found metabolic rate to be similar during exercise at a given absolute power output between menstrual phases (Dombovy et al. 1987; Hackney et al. 1991; Jurkowski et al. 1981) two studies have reported higher energy expenditure during exercise in the luteal phase compared to the

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follicular phase (Hessemer et al. 1985; Williams and Krahenbuhl 1997) (Refer to section 2.2.4 on metabolic rate).

Any condition that may alter the flux through the respective intermediate metabolic pathways, namely gluconeogenesis or aspartate and glutamate/glutamine transamination, will affect the acetate correction factor. For example fasting or starvation will increase flux through gluconeogenesis. Ketogenesis associated with fasting will not influence the correction factor as recycling of labelled acetate in ketones will be recovered, as ketones are not stored but are directly oxidised (Sidossis et al. 1995a). Increased protein catabolism may increase flux through specifically the alpha-ketoglutarate/ glutamate-glutamine reaction to form ketoacids as substrates for oxidation or gluconeogenesis (Wolfe 1992). Conversely, lipogenesis may include acetate as a substrate for fat synthesis and consequently overestimate label retention via the TCA cycle. However, evidence suggests that the inclusion of acetate in lipogenesis is negligible and in fact the proportion of FFA produced by de novo synthesis is small (Hellerstein et al. 1991).

1.3.3.4 Training status

Endurance training is known to increase mitochondrial density and hence TCA cycle enzyme activity, possibly in dissociation with the enzymes governing the TCA cycle exchange reactions. Therefore it is expected that training may increase the rate of recovery of CO_2 and thereby reduce label fixation resulting in a higher value for the acetate correction factor. Resting values can be expected to be higher in athletes due to the training-induced increase in basal metabolic rate. Exercise at a given relative intensity will occur at a higher absolute intensity in athletes and as absolute intensity is a determinant of the acetate correction factor (Shrauwen et al. 2000), the correction factor will be higher at any given percentage of VO_2max in athletes versus sedentary individuals. In addition, athletes generally have a lower percentage body fat and as this is also a major determinant explaining variability between individuals (Shrauwen et al. 2000), it further supports the argument for training status to influence the acetate correction factor.

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1.3.3.5 Substrate utilisation

Changes in the proportion of carbohydrate to fat utilised is a prime influence of variability in the acetate correction factor at rest and during exercise (Schrauwen et al. 2000). As mentioned previously (section 3.3.2, label position) fractional recovery of labelled CO_2 is higher when glucose is oxidised compared to fat as glucose produces more CO_2 per energy released (45.9 vs. 34.6 ml of CO_2 per kJ produced for CHO vs. fat, respectively) (Schrauwen et al. 2000). In addition the rate of energy production is greater when glucose is the fuel source compared to fat (1.0-2.4 vs. 0.4 mol (high energy phosphate) /min for carbohydrate vs. fat, respectively) (Jeukendrup et al. 1998), which would imply a faster rate of TCA cycling. Therefore with a greater ratio of carbohydrate to fat oxidised there is reduced chance of label fixation via the TCA cycle and thus a higher acetate correction factor. A change in the amount of protein utilised as an energy source will also influence the correction factor, as mentioned previously (section 3.3.3, metabolic state). An increase in protein catabolism will increase flux through the transamination exchange reaction with the tricarboxylic acid intermediate, alpha ketoglutarate (Wolfe 1992) and thus possibly increase label fixation. In this regard menstrual phase may once again be a confounding factor for the derivation of the acetate correction factor as many reports exist for changes in relative substrate oxidation with changes in the ovarian hormone concentration.

1.3.3.6 Gender

A gender difference exists in labelled acetate recovery at rest where the correction factor is significantly higher in women than in men (24.6 and 22.9% recovery respectively from a [1,2-^{13}C]acetate infusion) and remains different once corrected for variability in basal metabolic rate relative to fat free mass and RQ (Schrauwen et al. 2000). A significant gender difference also exists during exercise where the acetate correction factor is higher in men than in women (70.7 and 63.2% recovery, respectively from a [1,2-^{13}C]acetate infusion) but this difference falls away once corrected for differences in absolute energy expenditure relative to fat free mass and RER (Schrauwen et al. 2000).

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Therefore, considering all the variables that influence the acetate correction factor, particularly changes in metabolic rate and substrate utilisation, it appears prudent to consider the influence of the varying concentrations of ovarian hormones as they occur between menstrual phases on the acetate correction factor. Application of a “universal” acetate correction for correcting calculations of plasma FFA oxidation measured during different menstrual phases, when in fact the correction factor may vary with menstrual phase, will reduce the sensitivity of comparison. For this reason, we derived the acetate correction factor during three menstrual phases (i.e. EF, LF and ML phase) and these results are presented in the first study (Chapter 2) of this thesis.

Part 4

1.4 Summary

The literature to date demonstrates the multiple physiological effects of the ovarian hormones and occasionally identifies how the cyclical fluctuation of these hormones across a eumenorrhoeic cycle corresponds with changes in various physiological processes. The changes induced in metabolism per se, are possibly of the greatest consequence particularly when considering repercussions for exercise performance. Carbohydrate metabolism has been thoroughly investigated and ovarian hormone-induced changes to whole body glucose kinetics, contraction-stimulated glucose uptake, insulin resistances and glycogen or overall carbohydrate use have been reported. Speculations of an oestrogen-stimulated increase in overall fat use and animal research findings of increased fat mobilisation with oestrogen treatment have not been thoroughly followed up with tracer kinetic studies in eumenorrhoeic women (**Chapter 3**). The availability and validation of the use of stable free fatty acid tracers allows for the design of such studies. However, the hydrophobic nature of free fatty acids necessitates the use of a carrier, routinely human serum albumin, for the parenteral delivery of the tracer and this may pose some obstacles. The viability of using alternative carriers for FFA tracers should be explored (**Chapter 4**). Furthermore, application of the currently accepted acetate correction factor in calculations of plasma FFA oxidation may require reconsideration because of possible variability imposed on this correction factor by menstrual phase (**Chapter 2**). Although some work has been

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done to identify menstrual phase variations in protein catabolism or turnover between menstrual phases, further work is justified, albeit protein catabolism contributes minimally to overall energy expenditure. The impact of the ovarian hormone-induced changes to physiological systems on endurance capacity and exercise performance at first glance appears inconsistent. However, closer consideration of the relative changes in oestrogen and progesterone between phase help to explain some of the inconsistency as progesterone appears to largely act in opposition to the many benefits afforded by oestrogen. In this regard, no study has considered the LF phase, which corresponds to the pre-ovulatory surge in oestrogen without any increase in progesterone, when assessing variations in endurance capacity or exercise performance (**Chapter 6**). By exception, oestrogen potentiates progesterone-induced increase in ventilation and the consequence of a persistent increase in ventilatory drive during prolonged endurance exercise on metabolic demand should be explored. Furthermore, consideration of possible relations between the change in ventilatory responses between menstrual phases and the actual ovarian hormone concentrations should be assessed (**Chapter 5**), instead of only simple between phase comparisons.