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

Oosthuyse, Tanja

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CHAPTER 3

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Naturally cycling oestrogen and progesterone influences plasma free fatty acid kinetics during exercise

Tanja Oosthuyse[1], David A. Gray[1], Bruce Davidson[1], Peter Apps[2], Dawn AJ Deftereos[1] and Andrew N. Bosch[3]

[1]School of Physiology, University of the Witwatersrand, Medical School, Johannesburg, South Africa; [2]Department of Bio/Chemtek, CSIR, Pretoria, South Africa; [3]Sports Science Institute of South Africa, University of Cape Town, Cape Town, South Africa

Running head: Exercising FFA kinetics and ovarian hormones

Corresponding Author:

T.Oosthuyse
School of Physiology
University of the Witwatersrand, Medical School
Postnet suite 19
Private Bag X8
Northriding 2162
Johannesburg
South Africa
E-mail: oosthuyse@polka.co.za
Tel: +27 11 794 5630
Fax: +27 11 643 2765

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ABSTRACT

Previous research suggests that oestrogen increases FFA availability during exercise and conversely, indicates the antagonistic influence of progesterone. Thus we determined whether plasma FFA kinetics, measured from a constant infusion of K^{+}[1-^{13}C]palmitate during 90 min of exercise at 60%VO_2max, is related to the ovarian hormone profile across the menstrual cycle. Eumenorrhoeic women (n=5) participated in two infusion trials during two phases of their menstrual cycle, i.e. the early follicular (EF) phase and either the late follicular (LF) (n=1), mid-luteal (ML) (n=3), or late luteal (LL) phase (n =1) to maximise the range of the ovarian hormones for correlation purposes. The increase in FFA concentration during exercise above EF phase values correlated positively (r=0.92, p=0.028) with serum oestrogen concentration. Furthermore, the change in palmitate rate of appearance (Ra) (r=0.86, p=0.06) and disappearance (Rd) (r=0.89, p=0.04) from the EF phase to second menstrual phase (LF/ML/LL) correlated positively with the serum oestrogen to progesterone ratio (E/P). Thus an elevated oestrogen concentration was associated with an increased FFA availability during exercise; however these responses tended to be modulated by progesterone and therefore dependent on the E/P ratio in the ML phase. Such variations in FFA availability may have implications for endurance performance in women.

Key words: Eumenorrhoea, menstrual cycle, fat metabolism, stable free fatty acid tracers, substrate utilisation, endurance exercise

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INTRODUCTION

The benefits of optimising substrate utilisation to suit the exercise modality is broadly known. The female sex-hormones, oestrogen and progesterone, have been implicated in having the capacity to alter substrate metabolism. Oestrogen is supposedly responsible for the increased fat utilisation during exercise and at rest in the late follicular [1] and mid-luteal phase [2-4] compared to the early to mid-follicular phase of the menstrual cycle. Furthermore, oestrogen is implicated in the overriding consensus of a gender difference in substrate utilisation during exercise, where women use more fat and less carbohydrate and protein as an energy source than men [5-7]. Indeed, animal studies have clearly demonstrated an oestrogen-stimulated increase in fat metabolism and concomitant glycogen sparing during exercise [8; 9]. Progesterone, however, is an oestrogen antagonist and when progesterone is present at high concentrations it may nullify the effects of oestrogen completely [8; 10-12]. Nevertheless, the increase in fat oxidation associated with elevated oestrogen concentrations may be a direct consequence of oestrogen increasing the activity of the key enzymes of fat metabolism [10] but may also be a result of an increased mobilisation of fat stores and hence an increased free fatty acid (FFA) availability.

It has been proposed that oestrogen increases lipolysis in fat stores by heightening the sensitivity of beta-adrenergic receptors to epinephrine [13] and/or by reducing lipolytic inhibition caused by the stimulation of alpha adrenergic receptors [14]. Oestrogen may also increase the lipolytic response by increasing the amplitude of the growth hormone (GH) secretion pulse at rest [15] and GH response to exercise [16]. Conversely, progesterone opposes the lipolytic effect of oestrogen by decreasing the amplitude of the GH secretion pulse [15]. Furthermore animal studies have found that oestrogen not only increases lipolytic rate in adipocytes, but encourages the redistribution of lipid stores to intramuscular fat stores [9; 17]. However, caution must be taken when extrapolating from observations made in animal studies as interspecies differences may occur in the regulation of lipid metabolism [18].

Considering the potential for an ovarian hormonal effect, it is surprising that the literature has not until recently contained reports on the influence of the ovarian hormones on plasma FFA kinetics during exercise, while the effects on glucose flux has been extensively addressed [2; 4; 12; 19]. Initially only two studies were published that compared plasma FFA flux by intravenous FFA tracer infusion, albeit only at rest, either during the follicular and luteal

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phase [20] or in postmenopausal women before and after oestrogen replacement [21]. Both studies report no significant difference in resting FFA flux between conditions or phases before experimental intervention. The induction of hypoinsulinemia in order to maximise lipolytic stimulation at rest also resulted in no variation in FFA flux between menstrual phases [21]. However, the presence of progesterone in the luteal phase may have precluded any oestrogenic effect from being identified. In the second study using postmenopausal women, Jensen et al. [21] imposed a hormonal clamp on insulin and GH at rest in an attempt to isolate the actions of oestrogen and showed a decreased rate of appearance (Ra) and rate of disappearance (Rd) of FFA with oestrogen-repletion. However, oestrogen acts partially by stimulation of the lipolytic hormone regulators and preventing this effect may produce a false scenario. Furthermore, it may be plausible to speculate that oestrogen's action on lipid mobilisation may vary from resting condition to exercise or epinephrine-stimulated where at rest it is possible that oestrogen promotes lipid storage to ensure availability of lipid as fuel for maternal use during times of metabolic stress (such as during exercise), with preferential glucose supply to be preserved pending the occurrence of a viable foetus.

It is only until very recently that menstrual phase comparative studies, conducted coincidently yet independently to the current study, have measured plasma FFA kinetics during moderate exercise and observed no significant difference between the early follicular (EF) and mid-luteal (ML) menstrual phases [22] or between the EF, mid-follicular (MF) and ML phases [23]. However, as oestrogen and progesterone often act in opposition to each other, some authors have suggested that the oestrogen to progesterone ratio (E/P) be considered in studies determining the metabolic influence of the ovarian hormones [8; 12]. Thus, although Jacobs et al. [22] and Horton et al. [23] found no difference in the group FFA kinetics from EF to MF and /or ML phase, these studies failed to consider individual differences in magnitude of increase in oestrogen and/or progesterone between menstrual phases and the interaction of oestrogen and progesterone in the ML phase based on the E/P ratio. We speculate that it may be possible for such between-menstrual phase group comparisons to falsely indicate the lack of effect of the ovarian hormones on exercising fat metabolism. Rather it may be essential to consider the individual's response to their current ovarian hormonal status in order to clarify the influence of these hormones on FFA kinetics.

Therefore, we aimed to investigate relations between changes in FFA rate of appearance and disappearance during submaximal exercise from one menstrual phases to another and the

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ovarian hormone profile and therefore chose to allow the participating women to be studied during different menstrual phases, instead of a strict comparison between say only two phases. Thus we considered the influence of the absolute oestrogen and progesterone concentration and their interactive affect based on the E/P ratio on plasma FFA turnover during exercise; as an individual's response to the circulating ovarian hormone milieu may be dependent on such factors and result in a different outcome than would be predicted from a between-phase group comparison of for example, EF verse ML phase.

METHODS

Five of the seven young healthy women who volunteered to participate in this study completed all experimental sessions (Table 1). All subjects were classified as sedentary, as none participated in any sport or followed a training program. All exercised for less than 2 hours per week. The women experienced regular menstrual cycles and had not taken oral contraceptives for at least 2 years. Subjects provided written consent to participate in all the procedures of this study after being informed of the purpose and possible risks. This study was granted ethical clearance by the Committee for Research on Human Subjects, University of the Witwatersrand, Medical School (M000521).

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Table 1. Subject characteristics

Characteristic	Mean ± SD
Height (cm)	163.4±6.3
Body mass (kg)	55.7±5.9
Age (years)	22.4±2.5
Percentage body fat	25.6±3.0
VO_2max (ml.kg^{-1}.min^{-1})	28.5±2.2
Exercise intensity (Watts)	46.6±12
Exercise intensity (%VO_2max)	61.4±3.5
Length of MC (days)	27.6±1.7
Day of ovulation	13.8±1.9

MC refers to menstrual cycle

PRELIMINARY TESTS

MENSTRUAL CYCLE CHARACTERISTICS

Subjects recorded their basal oral temperature each morning immediately after waking using a digital thermometer (Vital sign VS-10 Soar Corporation, Japan) accurate to 0.1^{o}C, in order to identify a biphasic temperature pattern that often characterises a eumenorrhoeic cycle. The timing of the luteinising hormone (LH) surge that induces ovulation was predicted using a home ovulation test (Clearplan, Unipath Ltd., Bedford, England).

MAXIMAL AEROBIC CAPACITY

Each subject completed a discontinuous VO_2max test on a cycle ergometer (Excalibur 911 900, Lode, Groningen, Netherlands) during the follicular phase (day 2-9). Published evidence supports that a similar VO_2max value is attained when using a continuous or discontinuous protocol [24]. We chose to use a discontinuous protocol as it is well-tolerated by sedentary subjects. On arrival, height, body mass and the sum of four skin folds (triceps, biceps, sub-scapular and supra-iliac) was measured for estimation of percentage body fat. After a 10 min

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warm up subjects performed 3 min exercise bouts starting at 100 Watts and increasing in increments of 20 Watts interspersed by recovery periods. During the recovery period subjects were allowed to rest for as long as they felt necessary (10-20 min) or until their heart rate returned to 100 beats.min^{-1}. During the 3 min exercise bout subjects breathed through a one-way valve mouthpiece that allowed them to inhale atmospheric air and all expired air was directed through a 3 L mixing chamber in a metabolic cart system (Oxycon-4, Mijnhardt, Bunnik, Netherlands). The oxygen and carbon dioxide analysers were zeroed using 100% nitrogen gas and calibrated using a commercially available gas mixture of known O_2 and CO_2 content and room air. The system calculated and produced readings of VO_2 and VCO_2 from conventional equations every 30 seconds. VO_2max was considered to have been attained when VO_2 differed by less than 1.5 ml.kg^{-1}.min^{-1} between successive workloads.

EXPERIMENTAL PROTOCOL

Each subject completed two sessions of 90 min of cycling exercise at 60%VO_2max during two different phases of the menstrual cycle. All five subjects performed one trial during their early follicular (EF) phase (2 to 8 days after the onset of menses) and a second trial during one other menstrual phase; either during the late follicular (LF) phase associated with the pre-ovulatory surge in oestrogen (two days preceding to day of positive LH surge) or during the mid-luteal (ML) phase (four to ten days after ovulation) or during the late luteal (LL) phase (four to one day preceding menstruation). The experimental trial for the second menstrual phase included these various menstrual phases so as to maximise the spread in the ovarian hormones between subjects for the purpose of identifying possible relationships between the change in metabolic response to exercise between menstrual phases (i.e. from phase 1 to phase 2) and the circulating ovarian hormone concentration. Each subject performed both trials at the same time of day and the ordering of the trials was randomised with respect to menstrual phase. The two experimental trials could not always be completed in the same menstrual cycle and in that case was completed over two successive cycles.

A resting blood sample was taken on the day of each trial for the measurement of serum progesterone and oestrogen concentration (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA), thereby confirming the subject's menstrual phase. The EF phase is characterised by a circulating oestrogen concentration between 37 and 250 pmol.L^{-1} and progesterone concentration lower than 3 nmol.L^{-1} and the LF phase corresponds to 360 –1377

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pmol.L^{-1} and less than 5 nmol.L^{-1} for oestrogen and progesterone concentration, respectively. In the ML phase the respective concentration ranges for the ovarian hormones are 220 to 955 pmol.L^{-1} and greater than 10 nmol.L^{-1} and in the LL phase 130-450 pmol.L^{-1} and less than 16 nmol.L^{-1}, for oestrogen and progesterone, respectively (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA). The intra-assay and inter-assay coefficients of variation for the oestrogen assay were 5.3% and 6.4%, respectively and 4.7% and 6.0% for the progesterone assay.

For 48 hrs before the first trial each subject kept a record of all their meals and was asked to follow it as closely as possible before the subsequent trial. Two hours before arrival at the laboratory subjects ate a packed meal (2062 kJ) consisting of a 175 ml yoghurt, 175 ml orange juice and 30 g granola bar, providing in total 74 g of carbohydrate, 14 g of fat and 12 g of protein. Subjects were asked to refrain from caffeinated foods and beverages on the day of each trial.

At the start of each trial, body mass was recorded and then a 20 G catheter was inserted into a vein on the subjects' right forearm for infusing, and a second 20 G catheter was inserted into the left forearm vein as close to the hand as possible for sampling. Subjects remained seated for 20 min with the hand to be used for blood sampling resting in a hot box set at 55 ^{o}C, in order to obtain arterialised-venous blood samples [25]. A 17 ml resting blood sample was taken and divided into various aliquots. Resting VO_2 and VCO_2 was measured for 4 min with the subject seated on the cycle ergometer (same as used for the VO_2max) and then subjects commenced cycling at 60% VO_2max. After 30 min of exercise a second set of blood samples and an expired air sample were collected and calorimetric measurements were made for 3 min. A priming dose of 5.5 µmol.kg^{-1} $NaH^{13}CO_3^-$ (98% enriched, Isotec Inc., Miamisburg, OH) was then given followed by a continuous infusion of potassium[1-^{13}C]palmitate in a 5% human serum albumin solution at a rate of 0.12 µmol.kg^{-1}.min^{-1}. All solutions were freshly prepared under a laminar flow hood one to two days before the experimental trial. The potassium [1-^{13}C]palmitate solution was always made up to yield a final concentration of 2.2 mM and the flow rate was altered for each subject depending on their body mass to maintain the correct infusion rate. The potassium [1-^{13}C]palmitate (98% enriched, Isotec Inc., Miamisburg, OH) was weighed and heated to 60 ^{o}C in a water bath before reconstituting it in a volume of heated sterile water that equated to 40% of the total volume. The tracer solution

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was reheated in the water bath before filtering it through a 0.2 µm Millipore filter into a 5% human serum albumin (HSA) solution. The 5% HSA solution was prepared from a 20 % HSA stock solution (SA blood transfusion bank, Johannesburg, RSA) by diluting the required volume of stock with saline and incubating it in the water bath before use. The HSA-palmitate solution was then mixed well and allowed to cool at room temperature. The solutions passed a standard test (LAL, Gel clot test, sensitivity 0.06EU) to ensure that they were endotoxin-free before use (GX99010, Coatest Gel-LAL, Chromogenix, Mőlndal, Sweden).

Blood samples were collected and calorimetric measurements were made every 15 min after the onset of the infusion. Subjects were permitted to drink water ad libitum. Subjects emptied their bladder before the placement of catheters and then a full urine sample was collected immediately after exercise for the measurement of urinary N_2 loss as an estimate of protein catabolism during exercise.

SAMPLE ANALYSIS

All blood samples were immediately stored on ice until centrifugation immediately after the 90 min experimental trial and the serum or plasma was stored at -20 ^{o}C for later analysis. A resting blood sample of 2 ml was collected into a vacutainer containing no additives and allowed to clot before centrifuging and serum was analysed for progesterone and oestrogen (17β-oestradial) by radioimmunoassay (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA). Five ml of blood was collected in tubes containing lithium heparin for catecholamine (epinephrine and norepinephrine) quantification by high performance liquid chromatography (HPLC). For catecholamine extraction from plasma, 0.5 ml of plasma was added to a 1.5 ml microcentrifuge tube containing 50 mg of alumina (A-1772, Sigma chemical Co, St Louis, MO, USA), 500 µl Tris buffer and 10 µl internal standard 3,4-dihydrobenzylamine (1ng/10µl) and was spun on a rotary wheel for 15 min. The sample was then washed twice with deionised water before eluting the catecholamines from the alumina with 200 µl perchloric acid. The catecholamines were separated by reverse phase HPLC (Varian 9001, Varian Associates, Sugar Land, Texas, USA) on an ODS Spherisorb RP-C18 (25cm x 4.66mm) column using as mobile phase a 0.1 M sodium octyl sulphate in 9% acetonitrile solution buffered at pH 3 with a monochloroacetic acid buffer and eluent detected by an electrochemical detector (Pulsed electrochemical detector, Waters 464, Millipore, MA, USA). Seven ml of blood was collected into EDTA tubes for plasma FFA concentration and

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enrichment analysis and glycerol concentration analysis. FFAs were extracted from plasma, isolated by thin-layer chromatography and converted to their methyl esters with boron triflouride in methanol using the method described by Wolfe [26]. Quantification of total FFA concentration and ratio of palimtate to total FFA was done by gas liquid chromatography (GLC) using a 10% SP 2330 packed column with a chromosorb 100/120 WAW support. An isothermal temperature setting was selected so that the column was at 195^{o}C with the injector and flame ionisation detector at 230^{o}C. Aliquots of samples containing FFA methyl ester derivatives were analysed for carbon-13 enrichment of palmitate by electron impact selective ion monitoring of the peak abundances of the mass-to-charge ratio 270 and 271 using a gas chromatograph-mass spectrometer (GCMS) by the Bio/Chemtek Department at the CSIR (Pretoria, South Africa). Plasma glycerol concentration was determined with a spectrophotometric method (cat. No. 148270, Boehringer Mannheim, Mannheim, Germany). Urine N_2 content was determined by quantifying urine urea and ammonia concentration by enzyme analysis and spectrophotometry (cat. No. 542946, Boehringer Mannheim, Mannheim, Germany).

CALCULATIONS

Non-steady state rate of appearance (Ra) and disappearance (Rd) was calculated based on the equations of Steele et al. and adjusted for stable isotopes [26]. Thus,

$$\text{Palmitate Ra } (\mu\text{mol.kg}^{-1}.\text{min}^{-1}) = \{F_i - pV[(C_2-C_1)/2] \times [(E_2-E_1)/(t_2-t_1)]\}/[(E_2+E_1)/2] - F \quad (1)$$

Where F_i is the infusion rate corrected for actual (or measured) % enrichment, F is the infusion rate of total palmitate (i.e. without correction for % enrichment). The effective volume of distribution (pV) for FFA is assumed as 40 ml/kg. C_1 and C_2 is the plasma palmitate concentration and E_1 and E_2 is plasma palmitate enrichment at the first and second time point, t_1 and t_2 respectively.

$$\text{Palmitate Rd } (\mu\text{mol.kg}^{-1}.\text{min}^{-1}) = Ra - pV(C_2-C_1)/(t_2-t_1) \quad (2)$$

Metabolic clearance rate (MCR) was calculated as follows:

$$\text{MCR } (\text{ml.kg}^{-1}.\text{min}^{-1}) = Rd/C \quad (3)$$

Where C is the average plasma palmitate concentration at t_1 and t_2.

Total FFA Ra and Rd was estimated by dividing palmitate kinetics by the fraction of palmitate to total FFA concentration.

Whole body substrate utilisation was estimated based on the non-protein RER (Wolfe 1992). Protein use (grams) was estimated by multiplying total urinary nitrogen excreted by 6.25. Total rate of carbohydrate and fat oxidation was calculated by:

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Carbohydrate oxidation ($g.min^{-1}$) = (4.55 x VCO_2) – (3.21 x VO_2) – (2.87 x n) (4)

Where n is urinary nitrogen excreted as grams per minute.

Fat oxidation ($g.min^{-1}$) = (1.67 x VO_2) – (1.67 x VCO_2) – (1.92 x n) (5)

Total amount of carbohydrate and fat oxidised (grams) was calculated by multiplying each calculated rate by the measurement time interval and then summing them together.

Total energy expenditure (kJ) was calculated by multiplying VO_2 by the factor 20.9 $kJ.L^{-1}$ and then multiplied by the measurement time interval and summed.

STATISTICAL ANALYSIS

Pearson's linear regression was used for identifying relationships between measured variables (such as metabolite concentration, FFA Ra/Rd, whole body substrate utilisation) and the logarithmic function of oestrogen and progesterone concentration, or the log ratio of oestrogen to progesterone (E/P), or the magnitude of increase in oestrogen (E-fold) or progesterone (P-fold) from the EF (i.e. the first menstrual phase) to second menstrual phase (i.e. LF/ML/LL). The regression analysis was performed firstly with the measured variable expressed in absolute values and data from both first and second menstrual phase included and secondly where the measured variable was expressed as a change from the first to second menstrual phase. In support of significant correlations, statistical analysis was performed between the EF and ML phase in the three participating subjects but is regarded as purely observational due to the severely limited sample size. That is, a two-way repeated measures analysis of variance (ANOVA) with Student Newman Kuel's post hoc test was used for comparisons (phase x time) between the EF and ML phase. Further observational comparisons were performed to compare the rate of change in plasma metabolite concentration over time between menstrual phases; the slopes from the line of best fit were compared using an unpaired t test. Results are presented as mean ± standard deviation (SD) and $p<0.05$ was considered significant.

RESULTS

VERIFICATION OF MENSTRUAL PHASES

All subjects successfully recorded the occurrence of an LH surge and their temperature charts reflected a biphasic temperature pattern, indicating that all subjects experienced ovulatory cycles (Table 1).

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Resting serum oestrogen and progesterone concentration confirmed that all subjects were in the correct menstrual phase (Table 2).

Table 2. Oestrogen (E) and progesterone (P) concentration at rest and the magnitude of increase in their concentration between menstrual phases

Subject	E ($pmol.l^{-1}$)	P ($nmol.l^{-1}$)	E ($pmol.l^{-1}$)	P ($nmol.l^{-1}$)	E/P	E-fold	P-fold
	EF			*ML*		*ML over EF*	
M	79	1.3	600	23.3	25.8	7.6	17.9
J	92	3.2	337	23.2	14.5	3.7	7.3
L	239	1.6	342	47.2	7.2	1.4	29.5
Mean ±SD	136.7 ±88.9	2.0 ±1.0	426.3 ±150.4	31.2 ±13.8	15.8 ±9.4	4.2 ±3.1	18.2 ±11.1
	EF			*LF*		*LF over EF*	
Z	111	2.1	873	3.5	249.4	7.9	1.7
	EF			*LL*		*LL over EF*	
W	249	0.5	222	9.5	23.4	0.89	19

E-fold, magnitude of increase in oestrogen above EF phase values; P-fold, magnitude of increase in progesterone above EF phase values; E/P, oestrogen to progesterone ratio in the second menstrual phase. EF- early follicular phase, ML- mid-luteal phase, LF- late follicular phase, LL- late luteal phase.

Our subject group presented with a broad range of ovarian hormones and with a large range in the magnitude of increase in oestrogen and progesterone between phases and a broad range of oestrogen to progesterone ratios in their “second phase” (LF/ML/LL) (Table 2), thus allowing trends between these ovarian hormone variables and metabolic responses to be investigated. The EF versus ML phase comparison is limited to the inclusion of only three subjects. However, for observational purposes it is interesting to compare the average response in these three subjects with the individual response of subject Z (in LF phase) and subject W (in LL phase) for the purpose of proposing hypotheses to be tested by future studies.

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PLASMA GLYCEROL AND FREE FATTY ACID CONCENTRATION

The change in glycerol from the first to second menstrual phase correlated positively with the logarithmic function of oestrogen (Figure 1a) but did not quite reach significance ($p=0.067$). In support of this near significant correlation, the observational between group comparison revealed that the rate of increase in glycerol over 90 min of exercise was different between menstrual phases (Figure 1b). That is, a comparison of best-fit regression lines of glycerol concentration over time revealed that the rate of increase (or slope) in glycerol was greater in the ML phase (2.2 ± 0.36 $\mu mol.L^{-1}.min^{-1}$) than in the EF phase (1.5 ± 0.19 $\mu mol.L^{-1}.min^{-1}$) ($p=0.003$). Moreover, the regression line describing the change in glycerol concentration from the first to the second menstrual phase (for example ML-EF and LF-EF) showed that the change in glycerol during exercise relative to the EF phase was significantly greater in the LF phase than in the ML phase ($p=0.02$) (Figure 1c). Thus observational findings support the correlation of a greater glycerol response with increasing oestrogen concentration.

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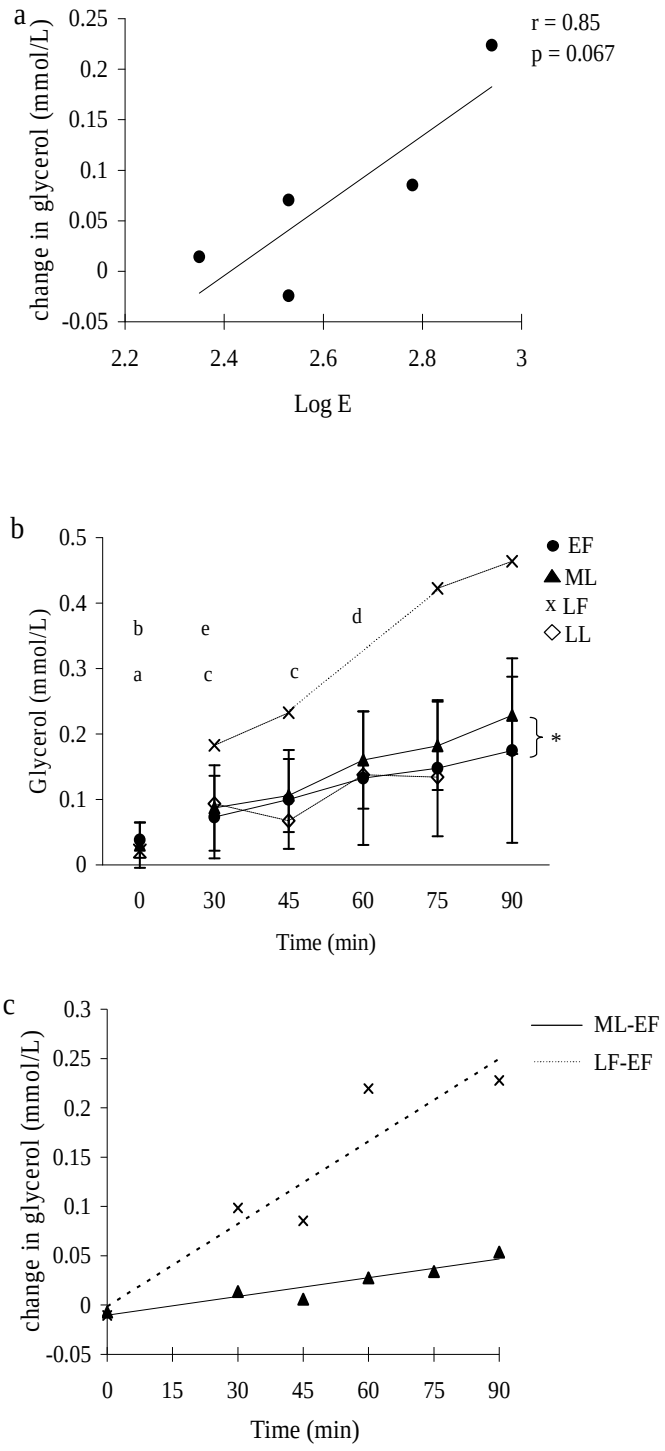


Figure 1: (a) The relationship between the change in glycerol concentration over EF phase values in all five subjects and log oestrogen (E) in the second menstrual phase. (b) Plasma glycerol concentration over time during exercise in the early follicular (EF) and mid-luteal (ML) phase (n=3), presented as mean and SD. Data for subject Z and W in their late follicular (LF) and late luteal (LL) phase respectively, are plotted for observational comparison. * Denotes a significantly greater rate of increase in glycerol concentration during exercise in the ML phase compared to EF phase, p=0.003. [a] 0 min EF less than 75 and 90 min EF (p<0.05); [b] 0 min ML less than all other time points ML (p<0.05-0.001); [c] 30 and 45 min ML less than 60, 75 and 90 min ML (p<0.05-0.001); [d] 60 min ML less than 90 min ML (p<0.05); [e] EF 30 min less than ML 30 min (p=0.03). (c) Comparison of the slopes of the regression line for change in glycerol concentration over the EF phase values in ML phase (n=3) and LF phase (n=1), p=0.02.

Resting plasma FFA concentration correlated strongly with the logarithmic function of oestrogen (r=0.82, p=0.013) and progesterone (r=0.72, p=0.043) concentrations. During exercise, we found a significant positive correlation between the average change in FFA

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concentration from the EF phase to the second menstrual phases (i.e. LF/ML/LL) and the logarithmic function of oestrogen in the second phase (r= 0.92, p=0.028, Figure 2a). These positive correlations are supported by the observational finding of a greater average plasma FFA concentration during exercise in the ML phase (1.3±0.46 mmol.L^{-1}) than in the EF phase (1.09±0.49 mmol.L^{-1}) (p=0.025) (Figure 2b).

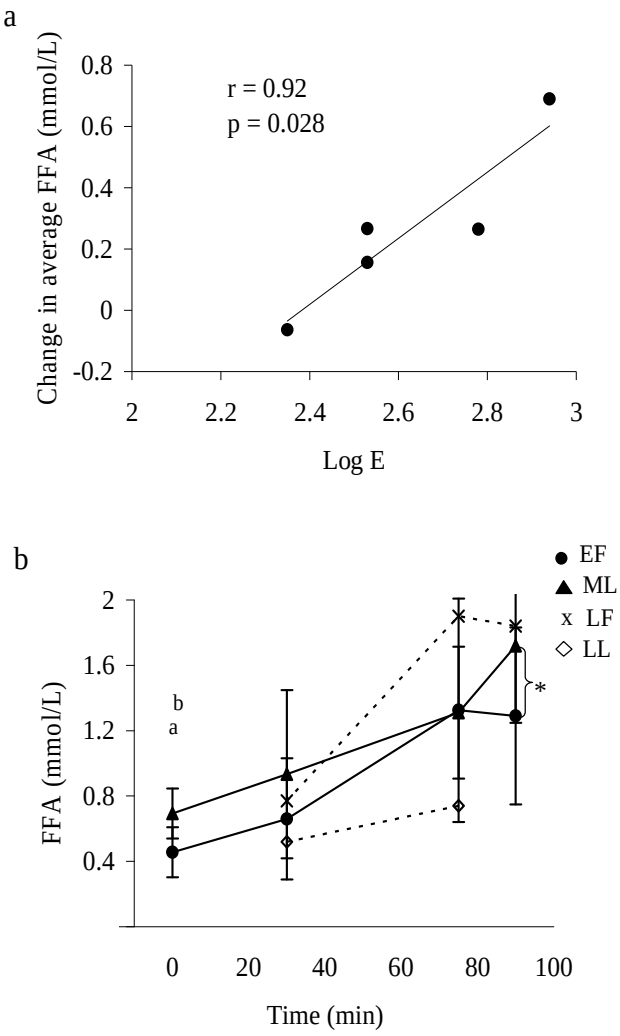


Figure 2: (a) Relationship of the change in average FFA concentration from EF phase and log oestrogen (E). (b) Plasma free fatty acid (FFA) concentration over time during exercise in the early follicular (EF) and mid-luteal (ML) phase (n=3), presented as mean and SD. Data for subject Z and W in their late follicular (LF) and late luteal (LL) phase respectively are plotted for observational comparison. [a] 0 min EF less than 75 min EF, p<0.05; [b] 0 min ML less than 90 min ML, p<0.05. * signifies significant difference between the average plasma FFA concentration during exercise EF and ML phase, p=0.025.

SYSTEMIC PALMITATE AND TOTAL FFA KINETICS

The change in palmitate Ra and Rd correlated positively with the logarithmic function of E/P in the second menstrual phase that was significant for palmitate Rd (p=0.044) and almost significant for palmitate Ra (p=0.064) (Figure 3a-b). Furthermore, the change in MCR

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correlated positively with the logarithmic function of the E/P ratio in the second menstrual phase but was not quite significant (p=0.063) (Figure 3c).

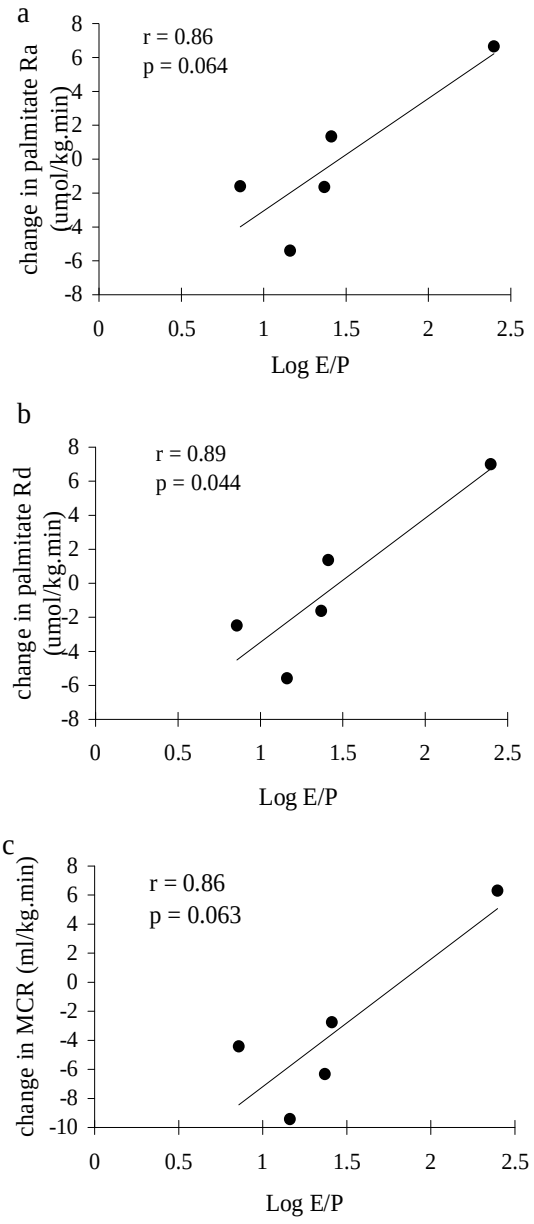


Figure 3: The relationship between the change in (a) Ra, (b) Rd and (c) MCR from EF phase values and log of the ratio of oestrogen to progesterone (E/P).

Although the data point corresponding to the highest E/P value looks like an outlier, there are no legitimate grounds on which to exclude the subject from data analysis and thus we maintain that the significant and near significant correlations identified represent true physiological relations. Furthermore, there was a tendency for the change in palmitate

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Ra/Rd/MCR to correlate with the logarithmic function of oestrogen (range of r=0.82-0.84, p=0.076-0.089) (where the data points appeared more evenly spread). Similar correlations were apparent when considering total FFA Ra, Rd and MCR (data not shown).

When calculating the difference in kinetics from menstrual phase 1 to 2 an observational trend is apparent (Figure 4a, b and c).

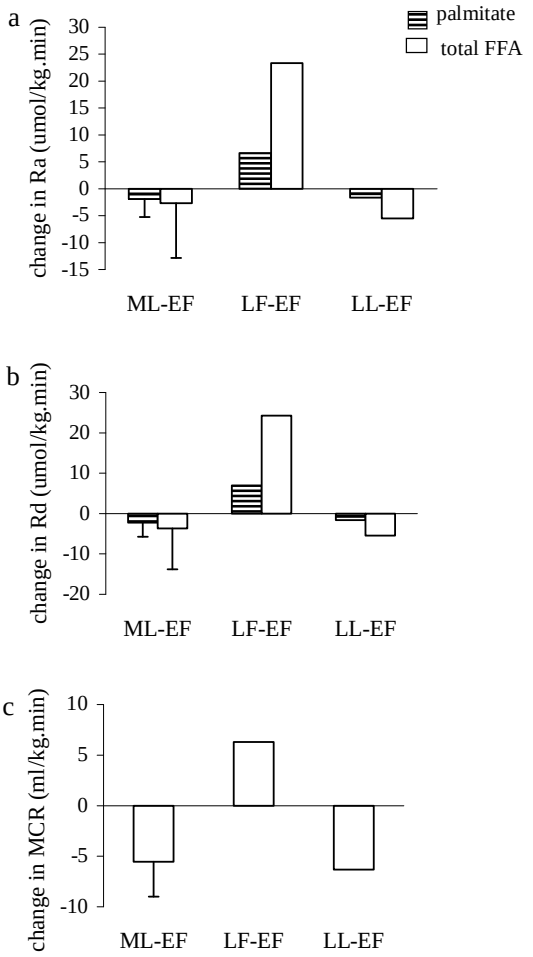


Figure 4: Observational comparison of the change in palmitate and total free fatty acid (FFA) (a) rate of appearance (Ra), (b) rate of disappearance (Rd) and (c) metabolic clearance rate (MCR) over EF phase values.

While the average change in palmitate or FFA Ra/Rd is slightly negative from the EF to ML phase and from the EF to LL phase (in subject W), a substantial increase in FFA and palmitate Ra and Rd occurred in subject Z from the EF to LF phase (Figure 4a and b). It is interesting to note that two of the subjects included in the EF versus ML phase comparison demonstrated a decrease in Ra and Rd from EF to ML and both of these subjects experienced only small increases in oestrogen from the EF to ML phase (1.4 and 3.7 fold, respectively)

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and they had a low E/P ratio (7 and 14, respectively) in the ML phase. The third subject included in the EF verse ML comparison demonstrated a small increase in Ra/Rd from the EF to ML and this subject experienced a larger increase in oestrogen from EF to ML (7.6 fold) and a much higher E/P ratio (25). In addition a noticeable drop in FFA MCR occurred from the EF to ML phase in all three subjects and in subject W from EF to LL phase, whereas an increase in MCR occurred from the EF to LF phase in subject Z (Figure 4c).Thus supporting the positive correlations identified. No significant difference was observed in the group comparison between EF and ML phase, albeit limited by sample size (Table 3).

Table 3. Systemic palmitate and total FFA kinetics in the final 15 min of a 90 min submaximal exercise session during various menstrual phases.

	EF (n=3)	ML (n=3)	LF (n=1)	LL (n =1)
Palmitate Ra ($\mu mol.kg^{-1}.min^{-1}$)	12.4±7.7	10.5±7.2	12.9	6.4
Palmitate Rd ($\mu mol.kg^{-1}.min^{-1}$)	12.5±8.2	10.2±7.2	12.9	6.4
FFA Ra ($\mu mol.kg^{-1}.min^{-1}$)	32.1±16.8	29.4±17.9	40.1	17.1
FFA Rd ($\mu mol.kg^{-1}.min^{-1}$)	32.4±17.9	28.7±17.9	40.2	17.1
MCR ($ml.kg^{-1}.min^{-1}$)	24.3±6.6	18.7±8.4^{+}	21.5	23.7
Ratio of serum [palmitate]/[tFFA]	0.37±0.05	0.34±0.03	0.32	0.37

Where appropriate values are presented as mean ± standard deviation. + indicates the development of a possible trend for a lower MCR in the ML phase compared to the EF phase (p=0.1). Ra and Rd refer to rate of appearance and disappearance respectively, FFA refers to free fatty acids or tFFA denotes total free fatty acids, MCR denotes metabolic clearance rate and [] indicate concentration

CATECHOLAMINE CONCENTRATION

No significant correlation was observed for epinephrine concentration or the change in epinephrine from menstrual phase 1 to 2 and the ovarian hormone parameters. No significant difference in epinephrine concentration was identified between the EF and ML phase at any time point, however limited by sample size (Figure 5a). Further observation of noticeably higher epinephrine levels in subject W in her LL phase remains obscure as this was the only trial conducted in the LL phase (Figure 5a). Conversely, norepinephrine concentration

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averaged over the last 30 min of exercise, correlated positively with the log oestrogen (Figure 5c) and log progesterone concentration (Figure 5d). Furthermore, norepinephrine concentration demonstrated a significant difference between the EF and ML phase at 90 min of exercise (Figure 5b), with higher levels in the ML phase ($p<0.05$).

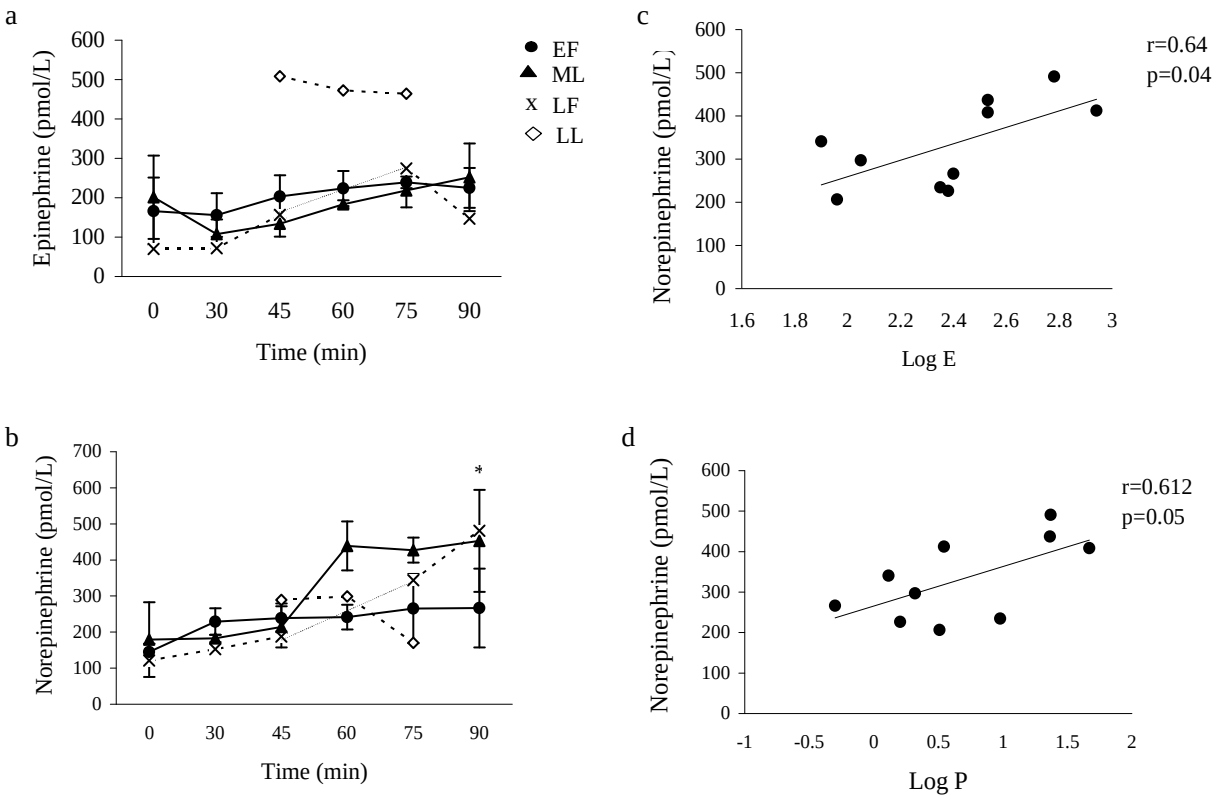


Figure 5: (a) Epinephrine and (b) norepinephrine concentration ($pmol.L^{-1}$) over time during exercise in the early follicular (EF) and mid-luteal (ML) phase (n=3), presented as mean ± SD. Data for subject Z and W in their late follicular (LF) and late luteal (LL) phase respectively are plotted for observational comparison. * Denotes a significant difference between EF and ML at that time point ($p<0.05$). The relationship between norepinephrine concentration and (c) log oestrogen (E) or (d) log progesterone (P).

WHOLE BODY SUBSTRATE UTILISATION

The change in the percentage contribution of carbohydrate to total energy expenditure from menstrual phase 1 to phase 2 was negatively correlated with magnitude of increase in progesterone from phase 1 to 2 (i.e. P-fold), such that the relation neared significance ($p=0.055$) (Figure 6b).

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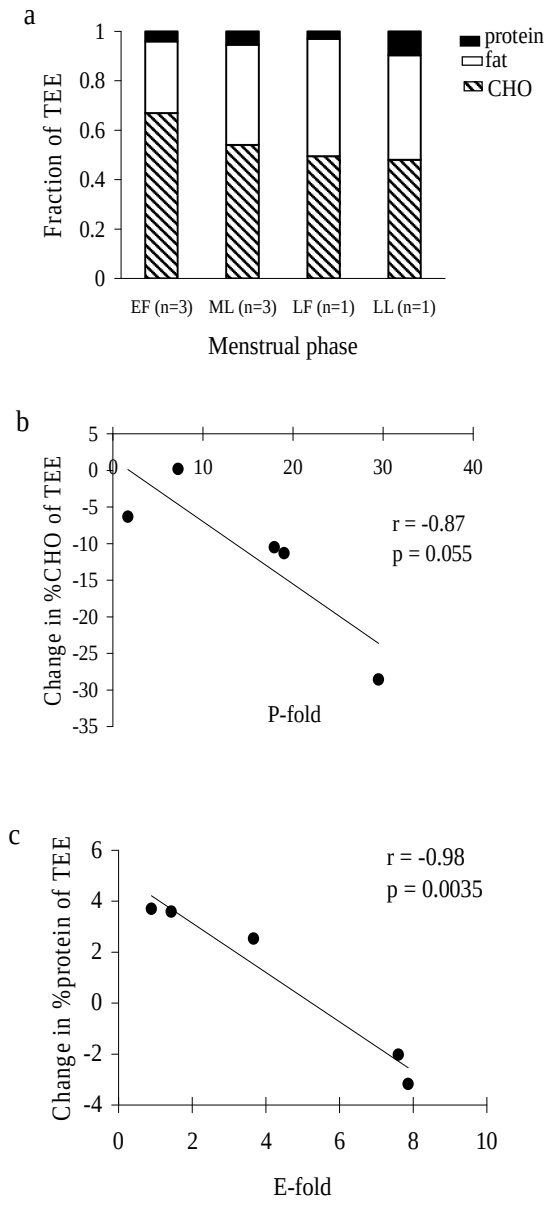


Figure 6: (a) Fractional contribution of carbohydrate (CHO), fat and protein to total energy expenditure (TEE). The relationship between (b) the change in the percentage of CHO contribution to TEE and the increase in progesterone (P-fold) from the EF phase and (c) the change in the percentage of protein contribution to TEE and the increase in oestrogen (E-fold) from the EF phase.

The total carbohydrate oxidised expressed as grams per kilogram decreased with increasing progesterone (p=0.023) (Figure 7).

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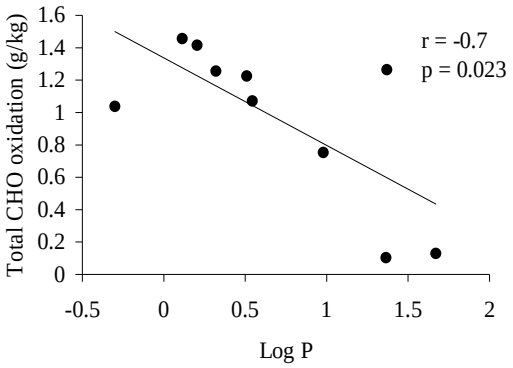


Figure 7: The relationship between total carbohydrate (CHO) oxidation and log progesterone (P) across the menstrual cycle.

In addition, the difference in the percentage contribution of protein to energy expenditure during exercise from phase 1 to 2 had a strong negative correlation with the magnitude of increase in oestrogen from phase 1 to 2 (i.e. E-fold) (Figure 6c) such that the percentage of protein making up TEE decreased with larger increases in oestrogen. Furthermore the absolute change in protein utilisation in grams from phase 1 to 2 had a significant negative correlation with the logarithmic function of oestrogen (Figure 8a), the magnitude of increase in oestrogen from phase 1 to 2 (E-fold) (Figure 8b) and the logarithmic function of E/P (Figure 8c). Observation of group comparisons, however limited by sample size revealed no significant difference in the fractional contribution of carbohydrate, fat and protein to total energy expenditure during exercise between the EF and ML phases (Figure 6a).

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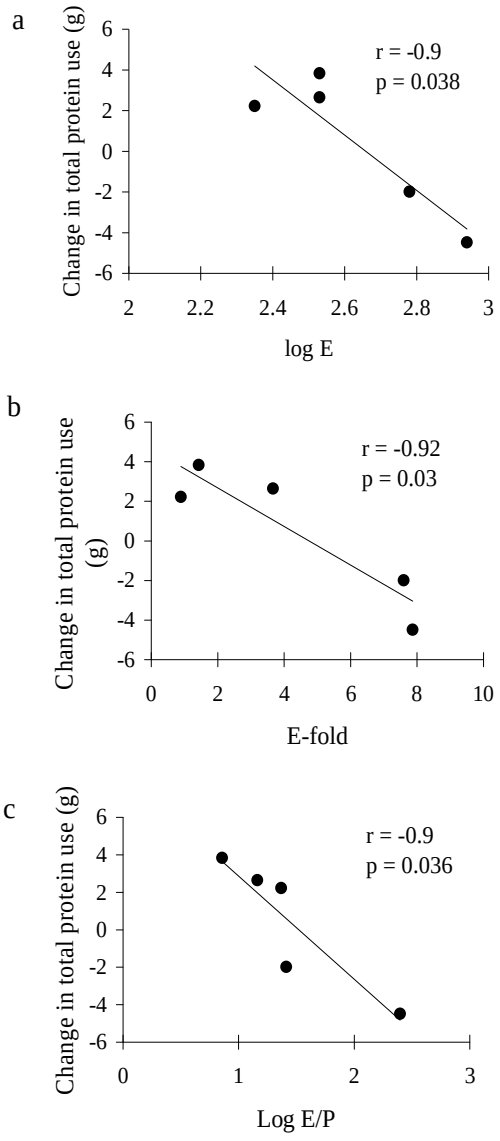


Figure 8: The relationship between the change in total protein use from the EF phase and (a) log oestrogen (E), (b) the increase in oestrogen over EF phase levels (E-fold), and (c) the log oestrogen to progesterone ratio (E/P).

DISCUSSION

The data presented by this current study is novel; no reports in the literature have considered the relationship between the concentration of the ovarian hormones or their interaction based on the E/P ratio and systemic FFA kinetics during exercise in eumenorrhoeic women. The first important finding of this study is that the positive correlations identified between the change in glycerol concentration, FFA concentration, palmitate kinetics and oestrogen concentration and/or the E/P ratio support previous suggestions of oestrogen's lipolytic properties and potential to increase availability of plasma FFA as an oxidisable source during

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submaximal exercise and the anti-oestrogenic influence of progesterone. Secondly, it is apparent that the magnitude of change in lipolysis and FFA availability during exercise from EF to ML phase is dependent on the E/P ratio as the concomitant increase in progesterone with the increase in oestrogen in the ML phase tended to modulate both the lipolytic and FFA kinetic response induced by oestrogen. Thirdly, the changes in the ovarian hormones correlated with whole body substrate utilisation. The effect on protein utilisation is especially interesting because no previous study that we are aware of has identified a relationship between elevated oestrogen concentration and reduced protein utilisation during exercise. However, despite the interesting relations identified in this study, interpretation of these findings are limited by the small sample size and therefore the results presented should be viewed as preliminary and warrant further studies with a larger sample size for verification.

Changes in glycerol concentration or the rate of glycerol appearance is often used as an indication of changes in lipolysis [26]. However, the underlying assumption of this association has been challenged [27; 28]. Nonetheless, many studies have compared blood glycerol concentration during submaximal exercise between the follicular and luteal phase of the menstrual cycle and most report no difference in the absolute concentration [4; 19; 29]. We are not aware of any study that has included the LF phase in the comparison. Furthermore, measurement of the glycerol response to exercise as the change in concentration over time may be a more sensitive method of determining differences in lipolytic sensitivity than simply comparing absolute concentration differences. That is, the observational comparison of the current study, revealed a greater rate of increase in glycerol concentration during submaximal exercise in the ML phase than in the EF phase, and to a significantly larger extent in the LF phase. This supported the near significant correlation ($p=0.067$) identified between the increase in glycerol above the EF phase values and oestrogen, suggesting a positive effect of oestrogen on lipolytic response during exercise. In contrast to the finding of the current study, Campbell et al. [2] found that glycerol concentration was lower in the luteal phase than in the follicular phase during submaximal exercise in the fasted state. However, the lower glycerol concentration in the luteal phase in the study of Campbell et al. [2] may be related to the very high progesterone concentrations reported in these subjects during this phase (40 $ng.ml^{-1}$ or 127 $nmol.L^{-1}$), thus demonstrating a possible anti-lipolytic effect of progesterone. The lower rate of increase in glycerol concentration during

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the ML phase compared to the LF phase observed in the current study would support this anti-oestrogenic effect of progesterone. A recent study considered glycerol turnover using stable tracers during exercise in the EF and ML phase and found no significant difference between these phases [30]. However, once again the anti-oestrogenic effect of progesterone may have nullified positive lipolytic affects of oestrogen in the ML phase in the study of Casazza et al. [30] and the E/P ratio should have been considered. The effect of the ovarian hormones on lipolysis is important, as lipolytic rate can be a determinant of plasma FFA oxidation rate [31].

Although some studies have reported lower concentrations of the lipolytic regulator, epinephrine, during exercise in conditions of increased oestrogen concentration [29; 32], like others [19; 33; 34], we found no influence of the ovarian hormone on epinephrine concentration. We did, however, find a positive correlation of exercising norepinephrine concentration with both oestrogen and progesterone concentration, supported by higher norepinephrine concentration after 90 min of exercise in the ML phase compared to EF phase. It is possible that the higher norepinephrine levels associated with greater oestrogen concentrations, could partly account for the significantly greater lipolytic response observed with increasing oestrogen levels. The concentration of other hormonal lipolytic regulators, such as growth hormone, insulin and cortisol were not measured.

FFA Ra is ultimately the difference between lipolysis and re-esterification and the capacity for the hydrolysed FFA to be released into circulation. Therefore, FFA Ra is considered a measure of FFA availability [6]. While total FFA Ra correlated with oestrogen and E/P ratio, the relations were the strongest with palmitate Ra, possibly because FFA Ra is an extrapolated value, whereas palmitate Ra was directly measured. The increase in palmitate and total FFA Ra from the EF phase to the second menstrual phase (LF, ML or LL) was most pronounced in the subject who happened to be in her LF phase with very high oestrogen and low progesterone levels, while the response in the ML phase appeared to be dependent on the E/P ratio. Nonetheless, oestrogen appears to promote the release of FFAs from endogenous stores firstly by increasing lipolytic rate (as discussed above) and secondly by possibly favouring the release of FFAs into circulation instead of uptake into adipocytes, as supported by the findings of Ellis et al. [17] who reported an oestrogen-associated decrease in the activity of adipose LPL with concomitant increases in plasma FFA concentration. Despite a noticeable modulation of palmitate or total FFA Ra with low E/P ratio in ML phase in the

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current study, the overriding positive effect of oestrogen on lipolysis was still observed in ML phase based on the glycerol response. Therefore, the tendency for a low E/P ratio to correspond with a decrease in FFA Ra may be a consequence of a secondary inhibitory effect of progesterone on aspects involving the release of hydrolysed FFA from the adipocyte; possibly changes in the rate of re-esterification or capacity to remove the hydrolysed FFA away from adipocytes.

The significant correlation between palmitate Rd or uptake and E/P ratio in the current study concurs with reports from animal studies of increased muscle triacylglycerol content with oestrogen therapy compared to placebo treatment and evidence of muscle TG storage even during submaximal exercise in the oestrogen treated group [9; 17]. However, the uptake of long chain fatty acids across the cellular membrane is in fact facilitated by fatty acid transport proteins [35] and the influence of varying ovarian hormone concentration on the content and kinetics of these transporters should be investigated. In particular, it would be interesting to consider the influence of the ovarian hormones on the fatty acid translocase FAT/CD36 in muscle following the recent report of 49% higher levels of this protein in women than in men, whereas no gender difference was noted in plasma membrane bound fatty acid binding protein (FABPpm) content [36]. The decrease in FFA Rd with a lower E/P ratio is most likely related to a relatively high progesterone concentration. That is, progesterone decreases FFA Rd possibly due to progesterone-induced decreased capacity for FFA oxidation by down-regulating the enzymes involved in cellular FFA oxidation [10] or possibly due to a decrease in the content and activity of the long chain fatty acid transporters. Progesterone has previously been shown to reduce the content of substrate transporters in skeletal muscle (i.e. GLUT4) [11].

Circulating FFA concentration is the overall result of lipolytic rate, rate of FFA release and uptake and therefore the changes observed in FFA concentration between menstrual phases and the positive correlation relative to the circulating oestrogen concentration follows a similar vein to the plasma glycerol and FFA kinetic data. An increase in resting and exercising FFA concentration with exogenously elevated oestrogen concentrations relative to placebo [32] or relative to combined oestrogen and progesterone supplementation [12] has been previously reported. The latter study again suggests the modulating effect of progesterone on oestrogen [12] and this is verified by other studies [2; 37] where FFA concentrations have been lower in the luteal phase than in the follicular phase during

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exercise, coincident with very high progesterone concentrations in the luteal phase (approx. 40 $ng.ml^{-1}$ or 127 $nmol.L^{-1}$). In the current study we found a significant positive correlation between resting FFA concentration and progesterone, but with a noticeable decrease in the degree of significance ($p=0.04$) compared to the relationship observed with oestrogen ($p=0.01$). Thus, as progesterone increased coincidently with oestrogen in the ML phase the positive correlation with progesterone may be more a reflection on the strong influence of oestrogen and the inability of progesterone to modulate this effect at the concentration present in our subjects. In fact, in the current study the average FFA concentration during exercise was significantly higher in the ML phase than in the EF phase (although only observational as limited by sample size) and is likely a result of the greater lipolytic response observed in the ML phase compared to EF phase together with the tendency for a lower MCR of FFA in the ML than in the EF phase.

Oestrogen promotes triacylglycerol esterification in skeletal muscle during exercise [17] and thus the larger uptake of FFA with increasing oestrogen is not necessarily all shuttled into beta-oxidation and may result in large intramuscular triacylglycerol stores. The effect of the ovarian hormones on the amount of plasma and intramuscular FFA used as oxidisable substrate during exercise has recently been compared between the EF and ML phase and no differences were observed between phases [22]. However, future studies should consider the effect of the E/P ratio in the ML phase and include the LF phase in their comparison of plasma FFA oxidation during exercise. Furthermore, our laboratory recently demonstrated that application of a menstrual phase specific acetate correction factor (which accounts for carbon label retention in the tricarboxylic acid cycle [38]) is essential when estimating plasma FFA oxidation rate using carbon tracers [39].

Indirect calorimetry is a crude estimate of substrate utilisation. Nonetheless, the changes in oestrogen and progesterone that occurred in the current study had an interesting effect on whole body substrate utilisation. Although no significant trend was identified with total fat oxidation per se, the decrease in carbohydrate oxidation with increasing progesterone supposedly demonstrates a shift toward less carbohydrate oxidation from EF to LF/ML/LL phase and not a progesterone effect per se. Our results also indicate a relationship between oestrogen and protein utilisation. Very high oestrogen concentrations reduce protein oxidation during exercise relative to low oestrogen levels of the EF phase. However, progesterone may stimulate an increase in protein utilisation relative to the EF phase. This is

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supported by a significant negative correlation between the change in protein utilisation over EF phase values and the E/P ratio. In addition, previous studies have documented increased protein utilisation in the ML phase compared to the EF or mid-follicular phase at rest [40] and during exercise [33; 41]. Following the findings of the current study we suggest the increase in protein use in ML phase of the latter studies is mediated via progesterone. In addition the previously documented gender difference in protein use during exercise, with less protein utilisation in women [5; 7], is possibly due to women being repeatedly exposed to elevated oestrogen concentrations.

SUMMARY AND RECOMMENDATIONS

The preliminary findings of this study suggest that oestrogen and progesterone (as they occur naturally across the menstrual cycle) influence the rate of FFA kinetics in the systemic circulation and by inference FFA availability during submaximal exercise; albeit with apposing actions. An oestrogen-induced increase in FFA availability, via an increase in mobilisation of lipid stores and cellular uptake, is expected to promote endurance capacity as FFA availability is regarded as one of the major determinants of the limits of FFA oxidation [42].

Therefore, the findings of the current study should serve as a caution to researchers to not only depend on group comparisons between menstrual phases when considering metabolic responses across the menstrual cycle, as is current practice. As a general guideline, based on the preliminary findings of our study, the LF phase or pre-ovulation period which coincides with a large spike in oestrogen will produce the most favourable metabolic response for endurance exercise with an increase in free fatty acid availability, while the first few days of the cycle (around menstruation) should coincide with the least favourable metabolic response for endurance exercise. However, the mid-follicular (roughly the second week of the menstrual cycle) and luteal phase (i.e. post-ovulation period- roughly the two weeks before menstruation) response will be expected to vary depending on the degree of increase in oestrogen and the relative increase in progesterone (in the luteal phase). That is, athletes who experience noticeable increases in oestrogen in the mid-follicular phase (as oestrogen is expected to gradually increase during this period), may experience greater FFA availability during exercise compared to say the early follicular response, while athletes with less of an increase in oestrogen during this phase will not enjoy this benefit. Although oestrogen also

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increases in the luteal phase, the magnitude of increase is variable, as is the magnitude of increase in progesterone which counteracts benefits afforded by oestrogen and hence the overall response in the luteal phase is dependant on both hormones. Thus, whether the benefits of oestrogen are evident during this latter phase will vary from individual to individual and from day to day. In addition, the possibility of an oestrogen inhibition and progesterone stimulation of protein utilisation during exercise should be studied further, as female athletes may need to compensate by increasing their protein intake in the luteal phase.

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