Original Article

Substrate oxidation in female adults during endurance exercise throughout menstrual cycle phases: IronFEMME pilot study

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ABSTRACT

The main aim of the study was to investigate the effect of menstrual cycle phases on substrate oxidation during steady state intensity exercise in adult females with regular menstrual cycle and on oral contraceptive (OC). Twenty-four healthy endurance and strength trained females, with regular menstrual cycle phases (n= 15; Age 35.6±4.2; height 163.9±5.9 cm; body mass 58.1±5.2 kg; VO_{2peak} 50.3±3.6 ml·min^{-1}·kg^{-1}) or on oral contraceptives (n=9; Age 30.4±4.5; height 163.9±9.0 cm; body mass 58.1±6.7 kg; VO_{2peak} 52.4±4.2 ml·min^{-1}·kg^{-1}) participated in the study. All participants performed a graded maximal exercise test to determine their peak oxygen consumption (VO_{2peak}). Participants then exercised at the speed corresponding to 75% of VO_{2peak} for 40 minutes on a treadmill in each menstrual cycle phase: regular menstrual cycle group (early follicular phase, mid-follicular phase and luteal phase) and OC group (hormonal phase and non-hormonal phase). There were no differences in the respiratory exchange ratio of each phase, in regular menstrual cycle phase group (mean±SEM): early-follicular phase 0.89±0.01, mid-follicular phase 0.87±0.01 and luteal phase 0.88±0.01 ($p>0.05$). There were also no differences in respiratory exchange ratio for the participants using oral contraceptive: hormonal phase 0.89±0.01 and non-hormonal phase 0.91±0.01 ($p>0.05$). However, we found that OC may influence fat oxidation ($p=0.018$) during the hormonal phase. Our preliminary results suggest that menstrual cycle and oral contraceptive do not influence substrate oxidation in females with regular menstrual cycle phases. Regarding the few disparities, more research is needed to understand how

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sexual hormones influence substrate oxidation in female. **Key words:** OXYGEN UPTAKE, FEMALE ATHLETES, ORAL CONTRACEPTIVES, RESPIRATORY EXCHANGE RATIO, ENERGY METABOLISM, OESTROGEN, PROGESTERONE.

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INTRODUCTION

The main energetic substrates used during endurance exercise are carbohydrates (CHO) and fats. Proteins represent 5-15% of the energy expenditure (Egan & Zierath, 2013). However, their involvement as fuel is considered to be negligible (Isacco, Duché, & Boisseau, 2012). The contribution of carbohydrate or fat to energy needs depends on: exercise intensity, mode and duration, nutrition and training status, age, and sex; and these factors should be taken into account when substrate metabolism is analysed (Isacco et al., 2012). In spite of the existing knowledge, data sources are few and conflicting specifically regarding substrate metabolism in athletic females (Kraemer et al., 2013; Oosthuyse, Bosch, & Jackson, 2005; Vaiksaar, et al., 2011). However, the majority of studies suggest females rely on more fat as a primary substrate during exercise than men. In particular, female endurance athletes throughout moderate-intensity exercise (Devries, 2015).

Differences across menstrual cycle phases due to natural fluctuations with the ovarian hormone may explain the sex differences observed in substrate oxidation (Constantini, Dubnov, & Lebrun, 2005; Isacco et al., 2012; Oosthuyse & Bosch, 2010). Oestrogens promote high muscle glycogen synthesis activity, which stimulate glycogen storage (Constantini et al., 2005). Oestrogens also stimulate lipolysis and increase the availability of plasma free fatty acids (FFA) (Dawson & Reilly, 2009; Oosthuyse & Bosch, 2010). Progesterone is suggested to oppose the oestrogen lipolytic effect (D'Eon et al., 2002). However, it emphasizes the carbohydrate sparing actions of oestrogens (Constantini et al., 2005; D'Eon & Braun, 2002). Therefore, the activity of both ovarian hormones on metabolism should be studied as a ratio of oestrogen/progesterone.

The use of the OC may interfere with substrate utilization during exercise. While modifications on metabolism influenced by OC are still controversial, there does seem to be consistency within dose and type of OC (Burrows & Bird, 2000; Lebrun, Joyce, & Constantini, 2013; Suh, et al., 2003). The main purpose of this work was to study the differences in substrates oxidation among hormonal phases in regular menstrual cycle (i.e. early follicular, mid-follicular and luteal phases) and OC users (i.e. hormonal and non-hormonal phases) in female endurance athletes, based on the respiratory exchange ratio (RER).

MATERIAL AND METHODS

Subjects

Twenty-four healthy endurance and strength trained females participated in this study. Fifteen females with regular menstrual cycles (n=15) and nine females taking OC (n=9). Descriptive data shown in table 1. All the females in OC group consumed stable monophasic. Before any test, informed consent was obtained after a description of the study as well as detailing out any risks and/or benefits. The ethics committee of Technical University of Madrid approved this study. All participants were healthy female adults between the ages of 25 and 40 years old who trained for 5 to 12 hours per week. "Training" included endurance sports with at least one-year experience in strength training. Excluded from the experiment were any female who: were not free of iron deficiency (serum ferritin >20 µg/l, haemoglobin >115 µg/l and transferrin saturation >16%), had thyroid problems or metabolic disease, consumed medication or had dietary supplements that alter vascular function, were pregnant, had undergone ovariectomy or were smokers.

Screening protocol

Body composition, including height (stadiometer SECA) and body mass (Beurer GmbH Germany bascule), was analysed for all participants during the first visit to the laboratory. Body composition analysis was performed with a Dual-Energy X-ray Absorptiometry (DEXA), which measures body fat mass (%), total body

fat mass (kg) and free fat mass (kg), using a GE Lunar Prodigy apparatus (GE Healthcare, Madison, Wisconsin, USA). Resting blood samples were obtained in an early morning fasted state after which participants performed a graded maximal exercise test. The maximal graded test was performed with a computerized treadmill (H/P/COSMOS 3PW 4.0, H/P/Cosmos Sports & Medical, Nussdorf-Traunstein, Germany) to determine each subject's VO_{2peak}. Expired gases were measured breath-by-breath with the gas analyser Jaeger Oxycon Pro (Erich Jaeger, Viasys Healthcare, Germany). Heart response was continuously monitored with a 12-lead ECG. Participants began with a warm-up of 3 minutes. The speed was increased to 0.2 km/h every 12 seconds. A slope of 1% was set throughout the test. This test was carried out in the early follicular phase (between days 2 and 6 of a normal cycle) for the regular menstrual cycle group, or in the non-hormonal phase for the OC group. The result of this test was used to determine the work intensity needed to obtain the speed corresponding to 75% of VO_{2peak} for the steady state running tests.

Experimental protocol

Once participants were screened, all performed the same experimental protocol using a treadmill, in each hormonal phase. The regular menstrual cycle group performed the protocol for three different phases (early follicular phase, mid-follicular phase and luteal phase) while OC group performed twice (hormonal phase and non-hormonal phase) (figure 1). The dates to perform each experimental control were calculated by a gynaecologist and were based on the participants' previous recorded cycles (four in total).

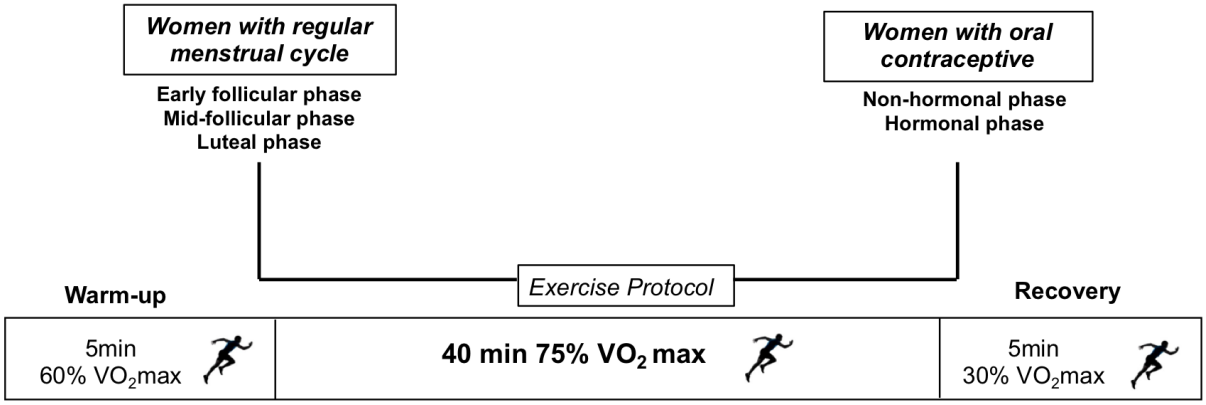

Figure 1. Experimental protocol diagram in each menstrual cycle phase.

Subjects arrived at the laboratory at 07:00 am on test day. Participants abstained from alcohol, caffeine, and exercise for 24 hours prior to the laboratory visit. They had breakfast 2 hours before arriving at the laboratory and composition breakfast was controlled and supervised by a nutritionist in order to avoid pro-inflammatory food. They were asked to have breakfast at the same time and the same kind of meal. Prior to the test, weight and blood pressure were recorded, and blood samples were taken by a nurse for later confirmation of the menstrual cycle phase. Arterial blood samples were collected via the radial arterial catheter and were immediately frozen at -80ºC before their delivery to the clinical laboratory (in 1-15 days). Blood biochemistry and determination of estradiol and progesterone were measured with standard technique. After this, one of the main investigators explained again in detail the stable running test protocol. This consisted of a 5 minute warm-up at the speed corresponding to 60% of VO_{2peak}, followed by 40 minutes of continuous run at the speed corresponding to 75% of VO_{2peak}, and finishing with a 5 minute recovery period at the speed corresponding to 30% of $VO_{2peak.}$ During the test, oxygen uptake (VO_2) and carbon dioxide (VCO_2) were measured continuously. The relative rates of whole body carbohydrates (CHO) and fat oxidation (FAT) were calculated. RER ranges from 0.7 (fat is the main oxidised substrate) to 1.0 (carbohydrate is the main oxidised

substrate). We assumed that urinary nitrogen excretion rate was negligible. For statistical analysis, RER, CHO oxidation (g/min) and fat oxidation (g/min) were average every 5 minutes during the main part of the experimental protocol (i.e the 40 min at 75% VO_{2peak}).

Dietary control

All participants received instructions to complete a 72-hour diet-record before and during the experimental days and were given healthy nutritional recommendations. The instructions given to the athletes were to avoid refined carbohydrates, fried foods, soda and other sweetened beverages, salty snacks, ice cream, margarine, processed meat and precooked foods. Participants were asked to replicate their diet prior to each trial.

Statistical analysis

A non-parametric Friedman ANOVA was used to analyse differences among menstrual cycle phases (early follicular, mid-follicular and luteal phase for regular menstrual cycle group). Wilcoxon test was applied to test differences between hormonal and non-hormonal phase for OC group. These statistical tests were conducted on RER, CHO oxidation and fat oxidation. Secondly, Cohen effects sizes (ES) were calculated to verify the magnitude of the mean differences between menstrual phases. The ES were interpreted based on the following criteria: <0.2 = trivial, 0.2 to 0.6 = small effect, 0.6 to 1.2 = moderate effect, 1.2 to 2.0 = large effect, and >2.0 = very large (Hopkins, 2006). The 90% confidence interval (CI) was also calculated. Magnitude-based inferences were carried out to determine the beneficial, trivial, or harmful effect of the menstrual cycle phases. When a clear interpretation was possible, a qualitative inference was given as follows: 0.5% to 5%, very unlikely; 5% to 25%, unlikely; 25% to 75%, possibly; 75% to 95%, likely; 95% to 99.5%, very likely; and >99.5%, most likely (Batterham & Hopkins, 2006). SPSS version 22 (IBM; Armonk, NY, USA) and Microsoft Excel (Microsoft, Redmond, WA) were used to perform the statistical analyses. All tests were conducted with a 5% significance level ($p<0.05$).

RESULTS

Characteristics of the study subjects are shown in Table 1. There was no significant effect of menstrual cycle phase on RER in females with regular menstrual cycle. (χ^2= 3.459, p = 0.177; Figure 2A). CHO oxidation (χ^2=3.337, p=0.189; Figure 2B) and fat oxidation rates (χ^2=4.455, p=0.108; Figure 2C) were not significantly different between the menstrual cycle phases. The magnitude-based inference analysis suggested a likely effect of early follicular phase for RER, whereas there was a likely effect of this phase for fat oxidation (see Table 2).

Table 1. General characteristics and hormone concentrations of oestrogen and progesterone in menstrual cycle phases (early follicular, mid-follicular and luteal) and hormonal and non-hormonal phases.

Variable		Early follicular	Mid-follicular	Luteal
Regular menstrual cycle group (n=15)				
Age, years	35.6 ± 4.2			
Height, cm	163.9 ± 5.9			
Body mass, kg	58.1 ± 5.2			
BMI, kg/m^2	21.7 ± 2.2			
Body fat, %	24.2 ± 7.0			
Free Fat Mass, kg	42.0 ± 3.1			

VO_{2peak}, ml·min^{-1}·kg^{-1}	50.3 ± 3.6			
Estrogen, pg/ml		39.4 ± 18.4	82.7 ± 51.3	110.7 ±33.6
Progesteron, ng/ml		0.91 ± 0.79	0.53 ± 0.31	10.43 ± 5.58
Ratio		57.5 ± 33.4	276.8 ± 281.7	13.3 ± 6.5
OC group (n=9)		**Hormonal**	**Non-hormonal**	
Age, years	30.4 ± 4.5			
Heigh, cm	163.9 ± 9.0			
Body mass, kg	58.1 ± 6.7			
BMI, kg/m^2	21.7 ± 2.8			
Body fat, %	25.7 ± 6.7			
Free Fat Mass, kg	41.6 ± 5.6			
VO_{2peak}, ml·min^{-1}·kg^{-1}	52.4 ± 4.2			
Estrogen, pg/ml		23.2± 29.0	33.3± 27.0	
Progesteron, ng/ml		0.45± 0.19	0.45± 0.21	
Ratio		33.2± 9.6	81.1± 36.1	

n, number of individuals; OC group, oral contraceptive group; BMI, body mass index; VO2peak, peak oxygen uptake. Values are means± SD.

Table 2. Pairwise comparison for substrate oxidation in women with regular menstrual cycles. Results expressed as statistical significance, effect size and magnitude-based inference.

Variable		ES (90% CI)	P	Chances of being negative/trivial/positive	Qualitive inference
RER	Early vs Mid	-0.46 (-0.80, -0.12)	0.177¥	93.5/6.4/0	Likely
	Early vs Luteal	-0.46(-0.85, -0.08)		91.2/8.7/0.1	Likely
	Mid vs Luteal	0 (-0.32, 0.31)		10.7/79.9/9.9	Trivial
CHO oxidation	Early vs Mid	-0.24 (-0.53, 0.05)	0.189¥	61.3/38.5/0.2	Unclear
	Early vs Luteal	-0.27(-0.69, 0.15)		63.2/35.4/1.4	Unclear
	Mid vs Luteal	-0.03 (-0.4, 0.34)		17.9/71.0/11.1	Trivial
FAT oxidation	Early vs Mid	0.48 (0.16, 0.81)	0.108¥	0/4.3/95.7	Likely
	Early vs Luteal	0.49 (0.12, 0.87)		0/6.2/93.7	Likely
	Mid vs Luteal	0.01 (-0.27, 0.28)		6.6/84.9/8.4	Trivial

ES, effect size; CI, confidence interval; CHO, carbohydrate; Early, early follicular phase; Mid, mid-follicular phase; Luteal, luteal phase. ¥ Non-significant pairwais comparisons.

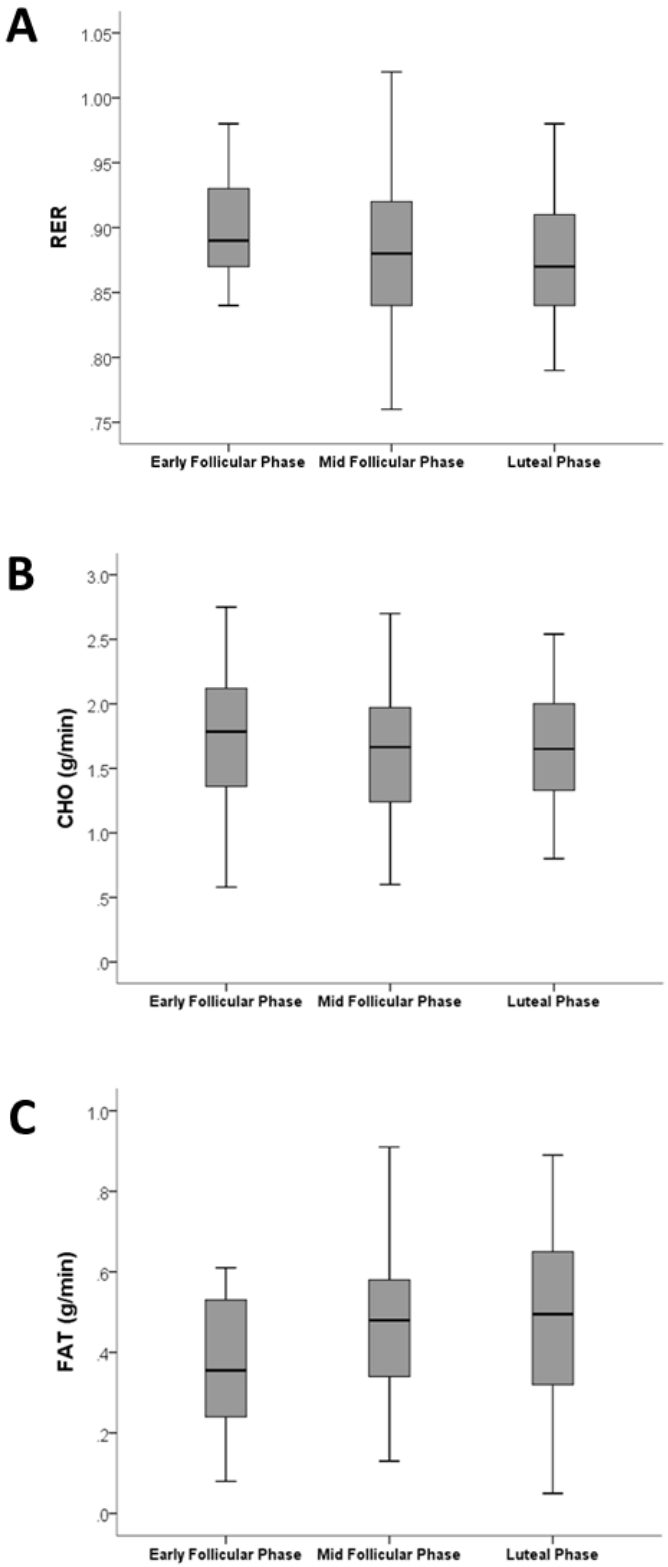

Figure 2. Substrate used during exercise at each menstrual phase in women with regular menstrual cycles. (A) Respiratory exchange ratio among different menstrual cycle phases in women with regular cycle. (B) Amount (g) of carbohydrates oxidized per minute during a 75% VO_{2max} running test. (C) Amount (g) of fat oxidized per minute during a 75% VO_{2max} running test.

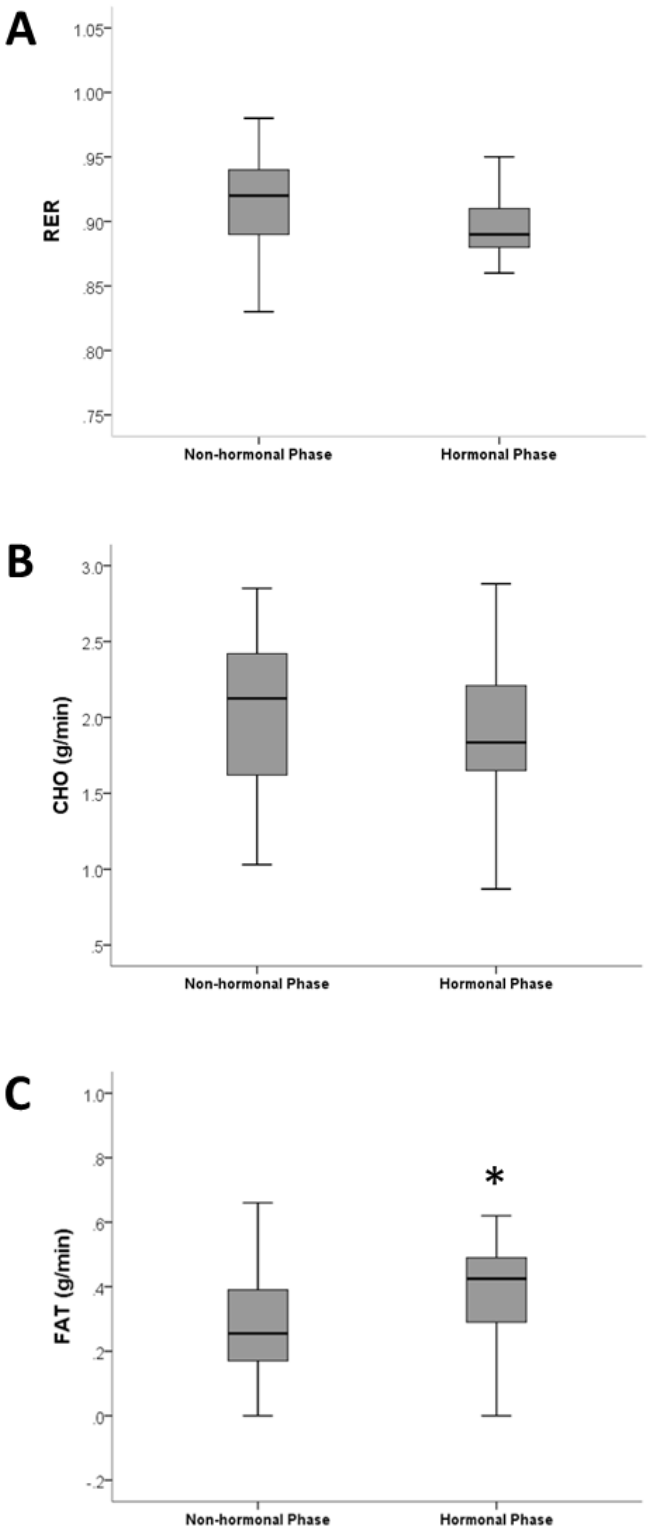

Figure 3. Substrate used during exercise at each menstrual phase in women on oral contraceptives pills. (A) Respiratory exchange ratio among different menstrual cycle phases in women on oral contraceptives pills. (B) Amount (g) of carbohydrates oxidized per minute during a 75% VO2max running test. (C) Amount (g) of fat oxidized per minute during a 75% VO2max running test. *Significant difference with non-hormonal phase.

In the oral contraceptive group there was not any significant effect of the menstrual cycle phases on RER (Z=-1.809, p=0.070; Figure 3A) and CHO oxidation (Z=-0.336, p=0.737; Figure 3B); whereas fat oxidation was significantly higher in hormonal phase (Z=-2.369, p=0.018; Figure 3C). The obtained ES and magnitude-based inference results suggested a possible effect of hormonal phase for fat oxidation (see Table 3).

Table 3. Pairwise comparison for substrate oxidation in women with oral contraceptive. Results expressed as statistical significance, effect size and magnitude-based inference.

Variable		ES (90% CI)	P	Chances of being negative/trivial/positive	Qualitive inference
RER	Non-Hormonal vs Hormonal	-0.38 (-0.96, 0.19)	0.07	74.0/23.6/2.4	Possibly
CHO oxidation	Non-Hormonal vs Hormonal	0.08 (-0.32, 0.48)	0.74	8.2/64.4/27.4	Trivial
Fat oxidation	Non-Hormonal vs Hormonal	0.29 (-0.13, 0.71)	**0.02**	1.2/32.0/66.8	Possibly

ES, effect size; CI, confidence interval; CHO, carbohydrate.

DISCUSSION

The present investigation analyses the differences in substrate oxidation among endurance and strength trained females characterized by a regular menstrual cycle or with taking OC. Despite previous equivocal evidence (Lebrun et al., 2013; Oosthuyse & Bosch, 2010), the present study found no differences in RER throughout menstrual cycle phases in either of the groups. This finding is in line with Vaiksaar et al. (2011) study, where they found no differences between RERs in follicular and luteal phases in regular menstrual cycle female rowers. The same authors studied females using OC (Vaiksaar et al., 2011) and achieved the same results. However, regarding the magnitude-based inference analysis made in this study, there is a likely effect in RER and fat oxidation when is compared early follicular phase *vs* mid-follicular phase and early follicular phase *vs* luteal phase. Therefore, these results in addition to the differences between studies still remain controversial.

The results of the present study could be explained by the number of factors influencing substrate oxidation during exercise. Among them are: nutritional status, oestrogen concentration relative to progesterone, other hormones, and the exercise intensity (Isacco et al., 2012). A possible explanation to our results is that participants were studied in a post-prandial state. Participants arrived at the laboratory 2h after they had breakfast. Consequently, they modified the substrate availability for the muscle cells by the time they performed the test. In turn, modifying the energetic environment of intra-myocellular and extra-myocellular determined the interaction between CHO and fat oxidation at a given exercise intensity (Spriet, 2014).

According to the scientific literature the metabolic fate of carbohydrates and fats change when oestrogens and progesterone arise in luteal and mid-follicular phases. During these phases, CHOs are stored as glycogen in liver and muscle, while fats become the primary fuel (D'Eon & Braun, 2002; Dawson & Reilly, 2009; Isacco et al., 2012; Lebrun et al., 2013). Furthermore, Suh et al. (2002) concluded that substrate availability and nutritional state (post-prandial *vs* fasting state) could be more determinant than sex hormone concentration in the selection of the energetic substrate during exercise in female athletes. This is in line with our results of a moderate effect on fat oxidation between early follicular phase and mid-follicular phase. These results suggest that ovarian hormones may have an effect on substrate oxidation but is not as important as

the influence of nutritional status. This moderate effect of oestrogen and progesterone may not be significant for female athletes who participate in recreational sports and are not on specific sport-diets; but it could make the difference in professional female athletes. We encourage those people who work with female athletes to take into account the menstrual cycle phases in their diets owing to the moderate effect shown in our study in recreational athletes.

In future research, this work suggests enlarging the number of participants studied, so the effects of the ovarian hormones in substrate oxidation can be further scrutinized. Regarding OC users, it was expected a lower RER during the hormonal phase. In fact, it is generally believed that OC users have a higher plasma FFA and cortisol concentrations, which means a higher lipolysis and lesser peripheral glucose uptake, and consequently a greater reliance upon fat as a fuel source may be expected (Beals, 2013; Devries et al., 2006). Despite the results of the magnitude-based inference analysis that appears to be a possible effect for fat oxidation (p=0.018) with higher values in the hormonal phase, our OC group did not show RER differences between hormonal and non-hormonal phases which is consistent with previous results (Suh et al., 2003). The OC influence on the type of fuel during exercise remains unclear because the interactions of OCs with energy metabolism are complex and there is inconsistency found in the literature (Lebrun et al., 2013). Another physiological parameter that might influence our results is the oestrogen/progesterone ratio, which D'Eon and Braun (2002) advocates that should be high enough to interact with the energy metabolism. This study found only a relatively high oestrogen/progesterone ratio indeed in mid-follicular phase, which is the same menstrual cycle phase where a moderate effect of two phases (early-follicular and mid-follicular) was found in substrate oxidation. The impact of sex hormones on other hormones such as catecholamines, insulin, cortisol and growth hormone (GH) (Beals, 2013; Comitato et al., 2015; De Crée, 1998; Kraemer, Francois, & Castracane, 2012), and the subsequent impact of those hormones on metabolism is another physiological factor that should be taken into account. Insulin and cortisol promote fat deposition both in experimental animals and in humans, while catecholamines and GH stimulate lipolysis (Comitato et al., 2015). Further, during exercise oestrogens modify insulin levels, which are higher in the luteal phase compared to the follicular phase (Kraemer et al., 2012). The differences in plasma insulin could impact carbohydrate metabolism, glucose uptake and total carbohydrate oxidation (D'Eon et al., 2002). According to D´Eon et al. (2002) CHO oxidation should be highest in luteal phase, however this was not reported in the present study. In the case of catecholamines, a greater epinephrine response to exercise (Kraemer et al., 2012) and lipolytic effect occurred while circulating oestrogen raised (D'Eon et al., 2002). Despite such lipolysis-boosting-effect of catecholamines the rise in oestrogen may not have been enough to activate lipolysis in the different subjects here studied. GH is affected by oestrogen too (De Crée, 1998; Kraemer et al., 2012; Leung et al., 2004). Exercise-induced GH secretion is greater during luteal phase and is accentuated at the same time than the exercise intensity (Kraemer et al., 2012). This study may deduce from the research listed above that, during luteal phases, due to a higher oestrogen concentration and a great GH amount, a lower RER at the same intensity should be obtained. However, no differences in RER throughout regular menstrual and oral contraception cycle phases were found. This could be due to the variability in the concentrations of hormones from one cycle to another as from day-to-day during any particular menstrual cycle phase (Oosthuyse & Bosch, 2010). Lastly, one of the prime physiological variables involved in the selection of the fuel source is exercise intensity (Isacco et al., 2012; Rapoport, 2010). We hypothesize that intensity could influence our results significantly. The intensity chosen to perform the experimental protocol in our work is the same as Knechtle et al. (2004) used in their experiment. Although they did not consider menstrual cycle phases, they got a RER of 0.86 ± 0.05, which means that fats were oxidized at the same rate as carbohydrates at 75% VO_2 peak (Knechtle et al., 2004). Our research found a higher RER in each of the menstrual cycle phase 0.89 ± 0.01, 0.87±0.01 and 0.88± 0.01, early-follicular phase, mid-follicular phase and luteal phase respectively and 0.91 ± 0.01 non-hormonal phase and 0.89± 0.01 hormonal phase, which

means that in all the phases, CHO where the preferential energetic substrate in both regular menstrual cycle and OC groups. In other research works, where the substrate oxidation was studied at 70% of VO_{2peak} in two different menstrual cycle phases (follicular and luteal phases) (Zderic et al., 2001) and two oral contraceptives phases (Vaiksaar et al., 2011), no differences in CHO and fat oxidation were found between phases. However, the conclusions from the study of Vaiksaar et al. (2011) are at odds with those of Burrows & Bird (2000), who state that the metabolic response varies from one to another within the oral contraceptive cycle. Moreover, they suggested that a chronic consumption of contraceptives might alter CHO and fat metabolism. The results from our study did show effect on substrate metabolism in OC users with a higher fat oxidation during hormonal phase ($p=0.018$). Above all, the disparities of results in regular menstrual cycle group as well as in OC group highlight the current situation: there is considerable intra-individual variability regarding the main substrate used as energy source during exercise at certain intensity.

CONCLUSION AND FUTURE PERSPECTIVE

No differences in RER throughout regular menstrual cycle neither in OC cycle were observed. These preliminary results suggest, along with previous studies, that daily fluctuations in hormones concentration would be the main factor determining the substrate metabolism, rather than the phase of the menstrual cycle, which has been determined by only one day. Moreover, in order to reduce the amount of intra-individual differences among the participants a control of the type and amount of macronutrients that compose the diet should be made, ovarian hormones as well as the other hormones, which might change the extra and intra environments of the muscle cell and, consequently, the substrate availability.

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