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Peak Fat Oxidation during Submaximal Exercise Remains Consistent across Menstrual Cycle and Combined Oral Contraceptive Phases

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ABSTRACT

LÖFBERG, I. E., J. E. KARPPINEN, I. LAATIKAINEN-RAUSSI, J. K. IHALAINEN, M. LEHTI, A. C. HACKNEY, and R. S. MIKKONEN. Peak Fat Oxidation during Submaximal Exercise Remains Consistent across Menstrual Cycle and Combined Oral Contraceptive Phases. *Med. Sci. Sports Exerc.*, Vol. 57, No. 7, pp. 1383–1394, 2025. **Purpose:** Substrate metabolism during exercise may vary across the menstrual cycle (MC) phases, likely due to estradiol (E2) and progesterone (P4). This study examined substrate metabolism during exercise in naturally menstruating (NoOC, $n = 34$) and women using combined oral contraceptives (COC, $n = 19$). **Methods:** Participants were measured in a fasted state in the follicular (FOL) and luteal (LUT) phases, or the inactive (INACT) and active (ACT) phases of COC use. Serum E2 and P4 were assessed using immunoassays and body composition via bioimpedance. Peak fat oxidation (PFO) and FAT_{MAX}, the intensity eliciting PFO, were evaluated using indirect calorimetry. FAT_{MAX} was calculated using peak oxygen uptake ($\dot{V}O_{2peak}$), measured on the following day. **Results:** PFO did not differ between FOL and LUT (0.40 ± 0.09 vs 0.41 ± 0.10 g·min^{-1}, $P = 0.482$) or INACT and ACT (0.48 ± 0.12 vs 0.44 ± 0.11 g·min^{-1}, $P = 0.099$). FAT_{MAX} showed no phase-related variation (NoOC: FOL 47.3% ± 15.7% vs LUT 47.7% ± 13.6%, $P = 0.727$; COC: INACT 57.1% ± 12.3% vs ACT 52.5% ± 12.2% $P = 0.172$). PFO was 0.08 g·min^{-1} (95% confidence interval [CI] = 0.02–0.14 g·min^{-1}, $P = 0.010$) and FAT_{MAX} 9.8% (95% CI = 1.0%–8.7%, $P = 0.031$) higher in the INACT versus FOL. The difference in PFO persisted after adjusting for fat-free mass and $\dot{V}O_{2peak}$ ($P = 0.033$) but was not significant after excluding an outlier from the COC group ($P = 0.108$). **Conclusions:** PFO and FAT_{MAX} remained stable between MC and COC phases, suggesting no need to standardize measurements by cycle phase. However, higher PFO and FAT_{MAX} in the COC group during INACT compared with FOL suggests distinct effects of exogenous hormones on metabolism compared with endogenous hormones. Practitioners should consider these differences when assessing factors influencing substrate metabolism. **Key Words:** FAT OXIDATION, CARBOHYDRATE METABOLISM, FEMALE PHYSIOLOGY, GRADED EXERCISE

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During exercise, fatty acids and carbohydrates are oxidized for energy in different proportions depending on various factors such as exercise duration and intensity (1), training status (2), nutrition (3), and potentially hormonal environment (4). Several studies have shown that women use more fatty acids and less carbohydrates than men at the same relative intensity of aerobic exercise (5–7), which is at least partly due to hormonal factors that affect energy production pathways (7). The most biologically active estrogen, 17β-estradiol (E2), has gained attention as a hormone that increases fatty acid availability (8,9) and stimulates fat oxidation (10), whereas progesterone (P4) is suggested to antagonize the metabolic effects of E2 (11).

E2 and P4 follow a predictable pattern during a normal menstrual cycle (MC) (12). Both hormones are at their lowest during the early follicular phase (FOL), with E2 levels gradually

increasing and peaking in the late FOL. P4 remains low during FOL but reaches its highest concentrations in the mid-luteal phase (LUT), approximately a week after ovulation. In the mid-LUT, E2 rises again, but to a lesser extent than in the late FOL (13). Indeed, several previous studies have reported higher fat and lower carbohydrate oxidation during submaximal exercise in the LUT compared with the FOL (14–16), but other studies have found no evidence of differences in substrate oxidation between MC phases (17–20) or have reported higher carbohydrate oxidation during the LUT (21). The interplay between E2 and P4 may be crucial in these metabolic changes, with noticeable alterations in substrate metabolism requiring a higher relative increase in E2 compared with P4 (22). However, despite the conflicting results in the field, the recent consensus indicates a lack of meaningful differences in substrate oxidation between MC phases. The recent systematic review and meta-analysis including 25 studies conducted by D'Souza et al. (23) stated that evidence is not strong enough to conclude that the MC phase has an effect on substrate oxidation.

The monophasic combined oral contraceptives (COC) provide a constant dose of exogenous female sex hormones throughout the usage period and are a commonly used type of contraceptive in physically active women (24). However, their effects on substrate metabolism during submaximal exercise are unclear. Some authors have reported higher fat oxidation (25) or lower carbohydrate oxidation with similar fat oxidation (26,27) in COC users compared with naturally menstruating women. During the 21- or 24-d active phase (ACT) of the COC cycle, endogenous E2 and P4 decrease to a level corresponding to the early FOL, followed by a slight increase during the 7- or 4-d pill-free inactive phase (INACT) (28). This downregulating effects of COC on endogenous female sex hormones together with the individual biological actions of synthetic ethinyl-E2 (EE) and progestin are thought to explain the reported metabolic differences between COC using and naturally menstruating women (28). Generally, evidence suggests that synthetic female sex hormones might have greater physiological actions than endogenous E2 and P4 (29,30). Synthetic EE is thought to have a higher binding affinity for estrogen receptors than E2, potentially leading to more pronounced physiological effects (31). Different progestins, in turn, have varying effects depending on their androgenicity (32), but in general, they are proposed to reduce enzymatic activity within glycolysis (30), which might lead to enhanced fat oxidation.

The lack of universal consensus on the effect of endogenous or exogenous female sex hormones on substrate metabolism during exercise might be partially due to different exercise protocols used in previous studies. Most studies investigating substrate metabolism across the MC and COC cycle have used prolonged single-stage exercise protocol (15–19,22,26,33). To date, only Frandsen et al. (20) and Isacco et al. (25) have investigated the influence of the MC phase or COC use on peak fat oxidation (PFO) using a graded exercise test developed to determine the intensity at which PFO occurs (34). However, these studies did not examine differences between COC phases (20,25). The aim of the present study was to investigate differences in PFO and carbohydrate oxidation between two hormonally verified MC phases, early FOL versus mid-LUT, and the INACT versus ACT of the COC cycle using a graded exercise test. In addition, we examined these potential differences in substrate metabolism between naturally menstruating women and COC using women.

METHODS

Participants. Seventy-seven healthy untrained women (tier 1: recreationally active, defined according to the participant classification framework) (35) 18–35 yr old with a self-reported body mass index (BMI) between 19.5 and 35 $kg \cdot m^{-2}$ volunteered to participate in this study. Participants were divided into two groups: 1) women with a self-reported MC length of 26–35 d in the past 6 months, not using hormonal contraception (NoOC group, $n = 58$), and 2) women using monophasic COC pills (COC group, $n = 19$). All participants met the inclusion criteria, which required that they were nonsmokers, not using medications affecting metabolism or exercise responses, and had no musculoskeletal disorders affecting running ability. The study adhered with the principles stated in the Declaration of Helsinki and was approved by the Ethics Committee of the University of Jyväskylä (1519/13.00.04/2021).

In the NoOC group, data from 34 participants were included in the analysis. Twenty-four participants were excluded for the following reasons: 1) incomplete data because of missing measurements due to illness or personal reasons ($n = 10$); 2) P4 concentration was below 16 $nmol \cdot L^{-1}$ in the LUT, indicating possible LUT deficiency (12) or scheduling error for assessment ($n = 11$); 3) the MC length of 38 d ($n = 1$); and 4) thyroid-stimulating hormone levels were above the reference range ($n = 2$).

The participants in the COC group used second-, third-, or fourth-generation COC pills for 21 or 24 d (ACT), followed by a 7- or 4-d hormone-free interval (INACT). The COC cycle length was 21 d of ACT and 7 d of INACT for 18 participants, whereas one participant had a COC cycle of 24 d of ACT followed by 4 d of INACT. Two participants used second-generation COC pills, seven used third-generation pills, and ten used fourth-generation pills. The specific preparations used by the COC group are detailed in a previous publication (36). All participants in the COC group were required to have been taking a COC for at least 3 months before the study. However, every participant included in the study had already been taking a COC for 5 months. There were no dropouts in the COC group during the study.

Study design. The study design was a part of the larger study "Women's Menstrual Cycle and Endurance Training (NaisQs)." The participants arrived at the laboratory on five occasions during the study, including the familiarization followed by two sets of measurements performed on consecutive days. In the familiarization session, the participants were introduced to the study methods and completed an informed consent and health questionnaire screened by a medical doctor. After familiarization, the participants started a control period lasting one MC or COC

cycle, during which they tracked their MC using a menstrual diary and a urinary luteinizing hormone (LH) surge test and reported physical activity in a training diary.

A three-step MC phase verification was performed according to the recommendations of Janse DE Jonge et al. (37). First, the measurements were preliminarily scheduled based on the participants' six previous cycles. Participants were instructed to use a hand-held electronic Clearblue two-hormone fertility monitors (Clearblue® Advanced Digital Ovulation, SDP Swiss Precision Diagnostics GmbH, Geneva, Switzerland) daily on an individually prescribed date depending on their MC lengths, following the manufacturer's instructions. If the MC length was varying, the use of testing device was based on the shortest cycle of the participant's six previous MCs. Tests were performed each morning using first morning urine, continuing daily until the result was positive. The testing device detects urine metabolites of estrone-3-glucuronide and luteinizing hormone (LH) to estimate the fertility window, providing readings categorized as "low," "high," and "peak" fertility. The first measurement was rescheduled to occur 4–8 d after a positive test result. LUT blood samples were retrospectively analyzed, and participants whose P4 concentration fell below 16 nmol·L^{-1} were excluded as previously described (12). The early FOL session was scheduled 1–5 d after the onset of menstruation. These phases were chosen to allow for a comparison between two distinct hormonal environments. The corresponding phases in the COC group were days 2–6 of the INACT (end of the COC cycle) and days 2–9 of the ACT (beginning of the COC cycle). Both measurement sessions were identical. Visits were conducted in a predetermined order to align with the subsequent training intervention, which started on the first day of the MC or COC cycle. Results from the training intervention will be published elsewhere.

The first measurement was completed after a 10-h overnight fast between 6:00 and 10:00 AM. The session included blood sampling, body composition assessment in minimal clothing with a multifrequency bioelectrical device (Inbody 770 Body composition analyzer, Biospace Co. Ltd, Seoul, Korea), and the PFO test. Height was measured on the first visit with a wall-mounted stadiometer. A peak oxygen uptake ($\dot{V}O_{2peak}$) test was scheduled for the day following the fasting session. Both $\dot{V}O_{2peak}$ tests occurred at approximately the same time of day to avoid diurnal variation. Participants were instructed to avoid strenuous exercise for the 24 h before fasting and performance measurements and to abstain from caffeine 4 h before both $\dot{V}O_{2peak}$ tests. Regarding nutrition, participants were advised to maintain their typical diet throughout the study period and avoid overeating or undereating on the day before each measurement session.

PFO test. The primary outcome was PFO, and the secondary outcomes were the rates of fat and carbohydrate oxidation relative to fat-free mass (FFM). The PFO test (Fig. 1) was performed walking on a treadmill (OJK-1, Telineyhtymä, Kotka, Finland), and respiratory gas exchange was measured breath-by-breath with Vyntus CPX metabolic cart (Vyaire Medical GmbH, Hoechberg, Germany). The indirect calorimetry device was calibrated according to the manufacturer's instructions and connected to the mask before each test.

The protocol development involved pilot testing with participants similar to the study's target group to ensure that the starting speed, intensity increments, and stage duration were suitable for accurately capturing PFO and FAT_{MAX}, defined as the intensity at which PFO was achieved (34). To achieve a steady state, we chose 4-min stages as recommended (38) and small increments of 1.2° in incline (34). We selected a starting speed of 4.5 km·h^{-1} to avoid unnecessarily prolonging the test duration. The speed increased by 1 km·h^{-1} from stage 1 to 2, and the incline increased by 1.2° from stage three onward until participants reached a respiratory exchange ratio (RER) of 0.95 (39). No verbal encouragement was given during the test.

To determine PFO, we used the measured-values approach (34), which has been shown to have a similar level of agreement

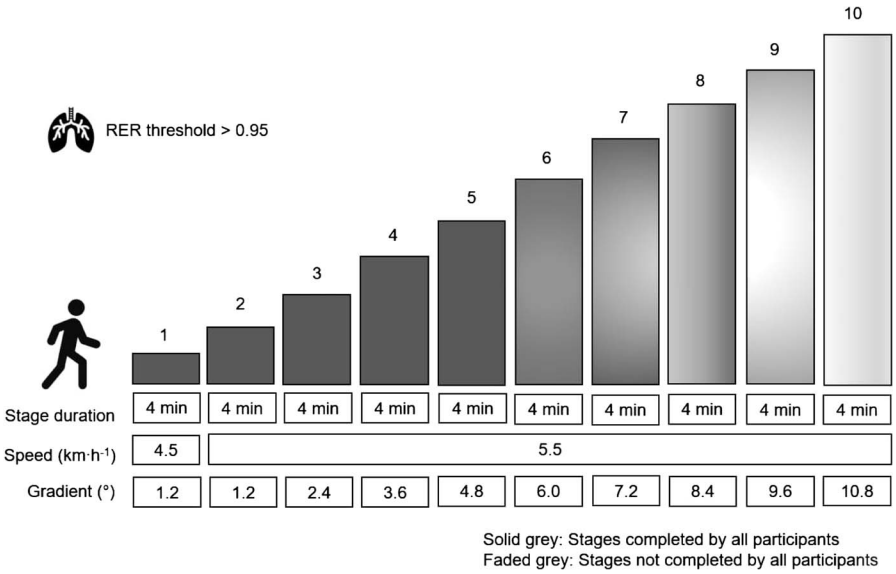

FIGURE 1—The schematic representation of the PFO test.

compared with other methods, such as polynomial modeling (40). In this approach, the breath-by-breath gas exchange data were averaged over 60-s intervals. Substrate oxidation was calculated for the last minute of each 4-min stage, using Frayn equation for fat oxidation (41) and intensity-dependent equations for carbohydrate oxidation (42), assuming negligible protein oxidation, as follows:

$$\text{Fat oxidation } (\text{g}\cdot\text{min}^{-1}) = 1.67 \times \dot{V}O_2 - 1.67 \times \dot{V}CO_2$$

$$\text{Carbohydrate oxidation } (\text{g}\cdot\text{min}^{-1}) \ (\leq 50\%\text{of } \dot{V}O_{2PEAK}) = 4.344 \times \dot{V}CO_2 - 3.061 \times \dot{V}O_2$$

$$\text{Carbohydrate oxidation } (\text{g}\cdot\text{min}^{-1}) \ (> 50\%\text{of } \dot{V}O_{2PEAK}) = 4.210 \times \dot{V}CO_2 - 2.962 \times \dot{V}O_2$$

The highest fat oxidation rate was selected as PFO. The corresponding $\dot{V}O_2$ value was used to calculate FAT_{MAX}, expressed as a percentage of $\dot{V}O_{2peak}$.

$\dot{V}O_{2peak}$ test. A graded treadmill test to determine $\dot{V}O_{2peak}$ was completed on the second measurement day using the same treadmill and metabolic cart. The $\dot{V}O_{2peak}$ test was performed on a separate day from the PFO test to minimize the risk of fatigue or fasting status affecting the $\dot{V}O_{2peak}$ results. Before the $\dot{V}O_{2peak}$ test, the metabolic cart was calibrated according to the procedure described above. The participants completed a short warm-up including a 3-min walk on a treadmill, followed by dynamic movements, a countermovement jump test, and a maximal bilateral isometric leg press test. The speed for the first stage was 6 $km\cdot h^{-1}$ and increased by 1 $km\cdot h^{-1}$ every third minute until volitional exhaustion. The incline remained at 0.6° during the test. $\dot{V}O_{2peak}$ was defined as the highest rolling average of 60-s $\dot{V}O_2$ using K-lab 3.1.25 software (2121.10.01 Aino Health Management Oy, Helsinki). Verbal encouragement was provided during the test.

Sex hormone measurements. Blood samples were collected from the antecubital vein on the fasting measurement, using 2 × 6 mL serum tubes (Vacuette® Tube Greiner Bio-One GmBH, Kremsmunster, Austria). The samples were left to clot for 15 min at room temperature before centrifuging for 10 min at 2245 g (Megafuge, 1.0R, Heraeus, Germany). The serum was separated and stored in a freezer (−80°C) for later analysis of E2 and P4 using chemiluminescence immunoassays on Immulite®2000 XPi-analyzator (Siemens Healthcare Diagnostics, Tarrytown, NY). Intra-assay coefficients of variation observed in our laboratory for E2 was 8.7% and for P4 15.5%, whereas analytical sensitivity for E2 was 55 $pmol\cdot L^{-1}$ and for P4 0.3 $nmol\cdot L^{-1}$. The specificity for EE was 20 $ng\cdot mL^{-1}$ with a cross-reactivity of 1.382%.

Statistical analysis. All statistical analyses were performed using SPSS Statistics 28 (SPSS Inc., Chicago, IL), and figures were graphed with GraphPad Prism (GraphPad Software, Boston, MA) and Microsoft® Excel® (Version 2404). The summary statistics are expressed as means ± SD or medians with interquartile ranges depending on the variable distribution. Normality was assessed using the Shapiro–Wilk test, histograms, and Q-Q-plots. For normally distributed data, within-group and between-group changes were analyzed by paired and independent samples Student's *t*-test, respectively. Non-normally distributed data were tested using the Wilcoxon signed-rank test and the Mann–Whitney *U* test. Pearson's correlation was used to assess the relationships between changes in PFO and changes in E2, P4, and E2:P4 ratio, with hormone levels log-transformed to normalize their distribution. The effect sizes are expressed as Cohen's *d* (43) or Hedges *g* (44). In between-group analyses, we chose to compare the early FOL to the INACT and the mid-LUT to the ACT. This decision is justified by the comparably low E2 and P4 during both early FOL and INACT phases, whereas the LUT and the ACT phases are characterized by large variations in endogenous hormone levels, as explained by Elliott-Sale et al. (28). To account for the potential confounding effects of FFM and $\dot{V}O_{2peak}$, we also performed the group comparisons using multiple linear regression with FFM and absolute $\dot{V}O_{2peak}$ ($L\cdot min^{-1}$) as covariates, as recommended (45). Because of the high variability of body composition in our sample, we examined association between PFO and adiposity by constructing a multiple linear regression model including FFM, $\dot{V}O_{2peak}$, and fat mass (FM) as covariates. These results are presented in Supplemental Figure 1 (Supplemental Digital Content, http://links.lww.com/MSS/D187). Statistical significance was set at $P \leq 0.05$.

In the COC group, we identified a potential PFO outlier in the INACT phase, with the value exceeding 1.5 × interquartile range, based on both visual inspection and statistical testing (46). This participant also had the highest PFO in the ACT phase, suggesting that the observation is credible and not due to measurement error. However, given that *t*-tests are sensitive to extreme values due to their reliance on the mean, we conducted a sensitivity analysis to assess the effect of this outlier. In this analysis, the participant was excluded when using PFO as the outcome measure.

Rates of fat and carbohydrate oxidation relative to FFM during the first four stages of the PFO test were compared between the MC and the COC phases expressed relative to exercise intensity (%$\dot{V}O_{2peak}$). $\dot{V}O_{2peak}$ values were missing for three participants in the FOL and two participants in the LUT within the NoOC group, as well as for two participants in INACT and one participant in ACT. Therefore, these participants were excluded from the analysis. Data from stages 5 to 10 were not included as the exercise intensity exceeded 75% $\dot{V}O_{2peak}$ for some participants, which is outside the valid range for assessing substrate oxidation using indirect calorimetry (42).

RESULTS

Participant characteristics. Data on baseline participant characteristics are presented in Table 1. The NoOC group was older than the COC group (mean difference 3.9 yr, 95% CI = 1.7 to 6.2 yr, $P < 0.001$), but both groups demonstrated similar BMI and height.

Table 2 displays the body composition, female sex hormones, and aerobic performance at FOL/INACT and LUT/ACT. In the

TABLE 1. Baseline characteristics in the NoOC (n = 34) and COC group (n = 19).

	NoOC	COC	*P* Value	*g*
Age (yr)	30.0 ± 3.8	26.1 ± 4.0	**<0.001**	1.0
Height (m)	1.66 ± 0.06	1.69 ± 0.05	0.083	0.51
BMI (kg·m^{-2})	25.1 ± 4.1	24.1 ± 2.1	0.330	0.03
Length of MC or COC cycle (d)	27 ± 2	21–24 + 4–7		

Results are presented as means ± SD.
NoOC, naturally menstruating; *g*, effect size (Hedge's). The notation 21–24 + 4–7 refers to the length of ACT and INACT of the COC cycle. Significant differences bolded ($P \leq 0.05$).

NoOC group, E2 and P4 levels were higher during LUT compared with FOL (E2: $P < 0.001$, $d = 1.75$; P4: $P < 0.001$, $d = 4.71$). However, the E2:P4 ratio was higher in the FOL compared with the LUT ($P < 0.001$, $d = 0.82$). In the COC group, E2 and P4 as well as the E2:P4 ratio remained stable across phases. Body mass was slightly higher during INACT compared with ACT (mean difference: 0.4 kg, 95% CI = 0.03 to 0.08 kg, $P = 0.037$), but the effect size was small ($d = 0.06$). No other significant phase-related changes were observed.

When comparing the two groups, E2 levels were higher in the NoOC group compared with the COC group in both the FOL ($P = 0.002$, $d = 0.39$) and the LUT ($P < 0.001$, $d = 1.80$). Correspondingly, P4 levels were higher in the NoOC group during the LUT ($P < 0.001$, $d = 4.76$). However, the E2:P4 ratio was higher in the COC group when comparing ACT to LUT ($P < 0.001$, $d = 1.08$). Absolute $\dot{V}O_{2peak}$ was higher in the COC group than in the NoOC group in the INACT ($P = 0.015$, $d = 0.83$), but not in ACT.

Substrate metabolism during two phases of the MC or COC cycle. PFO did not significantly differ between FOL and LUT (0.40 ± 0.09 vs 0.41 ± 0.10 g·min^{-1}, $P = 0.482$) or between INACT and ACT (0.48 ± 0.12 vs 0.44 ± 0.11 g·min^{-1}, $P = 0.099$) (Fig. 2). FAT_{MAX} values were also similar between both FOL and LUT (47.3% ± 15.7% vs 47.7% ± 13.6% $P = 0.727$) and INACT and ACT (57.1% ± 12.3% vs 52.5% ± 12.2%, $P = 0.172$). After excluding an outlier in the PFO data, the differences between INACT and ACT for both PFO and FAT_{MAX} were smaller (PFO: 0.46 ± 0.09 vs 0.43 ± 0.10 g·min^{-1}, $P = 0.172$, FAT_{MAX}: 56.9% ± 12.6% vs 52.1% ± 12.4%, $P = 0.173$).

Figure 3 illustrates fat and carbohydrate oxidation rates relative to FFM during the last minute of stages 1–4 at different relative intensities (%$\dot{V}O_{2peak}$) for FOL and LUT, and INACT and ACT. In the NoOC group, fat and carbohydrate oxidation rates were consistent across the four stages between the MC phases. In the COC group, fat oxidation relative to FFM was generally higher in the INACT compared with the ACT. Notably, in stage 1, the mean difference was 0.80 mg·kg FFM^{-1}·min^{-1} (95% CI = −0.03 to 1.63 mg·kg FFM^{-1}·min^{-1}, $P = 0.059$). A similar pattern was observed in stage 2, with a mean difference of 0.78 mg·kg FFM^{-1}·min^{-1} (95% CI = 0.00 to 1.56 mg·kg FFM^{-1}·min^{-1}, $P = 0.051$). Conversely, carbohydrate oxidation relative to FFM tended to be lower in the INACT compared with the ACT. In the stage 2, the mean difference was −1.39 mg·kg FFM^{-1}·min^{-1} (95% CI = −2.83 to 0.04 mg·kg FFM^{-1}·min^{-1}, $P = 0.056$).

Figure 4 displays the relationships between changes in absolute PFO values and changes in log-transformed E2:P4 ratio, E2, and P4 in both groups. In the NoOC group, there were no significant associations between changes in PFO and changes in the log-E2:P4 ratio ($r = 0.12$, $P = 0.492$), log-E2 ($r = 0.12$, $P = 0.494$), or log-P4 ($r = -0.04$, $P = 0.837$). The associations were weak in the COC group; changes in PFO were not significantly correlated with changes in hormone levels either before (log-E2:P4 ratio: $r = -0.34$, $P = 0.153$, log-E2: $r = -0.39$, $P = 0.103$, log-P4: $r = -0.13$, $P = 0.599$) or after exclusion of the PFO outlier (log-E2:P4 ratio: $r = -0.31$, $P = 0.218$, log-E2: $r = -0.31$, $P = 0.208$, log-P4: $r = 0.06$, $P = 0.817$).

Substrate metabolism between groups. PFO and FAT_{MAX} were higher in the COC group than in the NoOC group at FOL/INACT. The mean difference for absolute PFO was 0.08 g·min^{-1} (95% CI = 0.02 to 0.14 g·min^{-1}, $P = 0.010$), whereas the mean difference for FAT_{MAX} was 9.8% (95% CI = 1.0 to 18.7%, $P = 0.031$). In ACT, PFO was 0.03 g·min^{-1} (95% CI = −0.03 to 0.09 g·min^{-1}, $P = 0.372$) and FAT_{MAX} was 4.8%

TABLE 2. Body composition, female sex hormones, and aerobic performance at FOL/INACT phases and LUT/ACT in the NoOC (n = 34) and COC group (n = 19).

	Group	FOL/INACT	LUT/ACT	Within-Group (*P*, *d*)	FOL/INACT (*P*, *g*)	LUT/ACT (*P*, *g*)
Body composition						
Body mass (kg)	NoOC	68.9 ± 10.8	68.8 ± 11.0	0.823, 0.00	0.902, 0.04	0.801, 0.08
	COC	68.5 ± 6.7	68.1 ± 6.9	**0.037, 0.06**		
FFM (kg)	NoOC	48.8 ± 5.2	48.6 ± 5.2	0.129, 0.05	0.723, 0.10	0.728, 0.10
	COC	49.4 ± 4.5	49.1 ± 5.2	0.276, 0.06		
FM (kg)	NoOC	19.2 ± 5.5	19.0 ± 5.4	0.131, 0.03	0.692, 0.11	0.585, 0.16
	COC	20.0 ± 8.3	20.3 ± 8.6	0.591, 0.02		
Female sex hormones						
E2 (pmol·L^{-1})	NoOC	117 (76.5–175.3)	497.5 (413–667.5)	**<0.001, 1.75**	**0.003, 0.39**	**<0.001, 1.80**
	COC	56.9 (27.4–121)	72.3 (48.8–186)	0.494, 0.45		
P4 (nmol·L^{-1})	NoOC	1.3 (0.9–1.8)	27.1 (22.2–34.2)	**<0.001, 4.71**	0.129, 0.45	**<0.001, 4.76**
	COC	0.9 (0.5–1.4)	1.0 (0.8–1.3)	0.433, 0.09		
E2:P4 ratio	NoOC	80.3 (63.2–131.3)	17.8 (13.3–23.3)	**<0.001, 0.82**	0.054, 0.27	**<0.001, 1.08**
	COC	44.4 (30.8–131.0)	72.8 (58.4–174.3)	0.421, 0.29		
Aerobic performance						
$\dot{V}O_{2peak}$ (mL·kg^{-1}·min^{-1})	NoOC	37.7 ± 5.1	37.6 ± 5.2	0.626, 0.02	0.217, 0.38	0.183, 0.40
	COC	39.5 ± 4.7	39.6 ± 4.3	0.305, 0.00		
$\dot{V}O_{2peak}$ (L·min^{-1})	NoOC	2.6 ± 0.3	2.6 ± 0.3	0.580, 0.11	**0.015, 0.83**	0.138, 0.44
	COC	2.8 ± 0.2	2.7 ± 0.2	0.191, 0.19		

Results are presented as means ± SD or medians (interquartile range).
Within-group, the difference between MC or COC phases; FOL/INACT, the difference between NoOC and COC groups at follicular or inactive phase; LUT/ACT, the difference between NoOC and COC groups at luteal or active phase; *d*, effect size (Cohen's); *g*, effect size (Hedge's); E2, estradiol; P4, progesterone; FM, fat mass; $\dot{V}O_{2peak}$, peak oxygen uptake.
Significant differences bolded ($P \leq 0.05$).

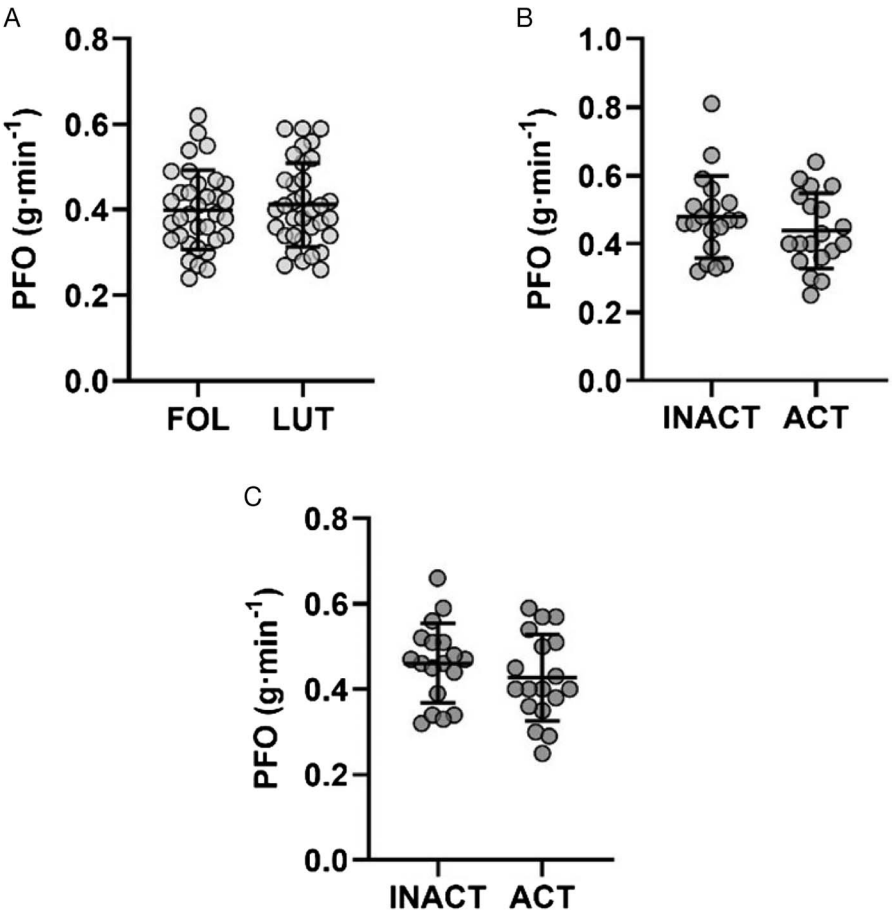

FIGURE 2—(A) PFO at FOL and LUT of the MC. (B) PFO at INACT and ACT of the COC cycle before the sensitivity analysis. (C) PFO at INACT and ACT of the COC cycle after the sensitivity analysis. Data are presented as means with SD and individual data points.

(95% CI = −2.9% to 12.6%, $P = 0.215$) higher compared with the LUT, but these differences were not statistically significant.

The difference in PFO between groups was smaller after excluding the participant identified as an outlier. Absolute PFO was 0.06 g·min^{-1} (95% CI = 0.01 to 0.12 g·min^{-1}, $P = 0.028$) higher in the COC group in the INACT compared with FOL, whereas the mean difference for FAT_{MAX} was 9.6% (95% CI = 0.5% to 18.8%, $P = 0.039$). In the ACT, PFO was 0.02 g·min^{-1} (95%

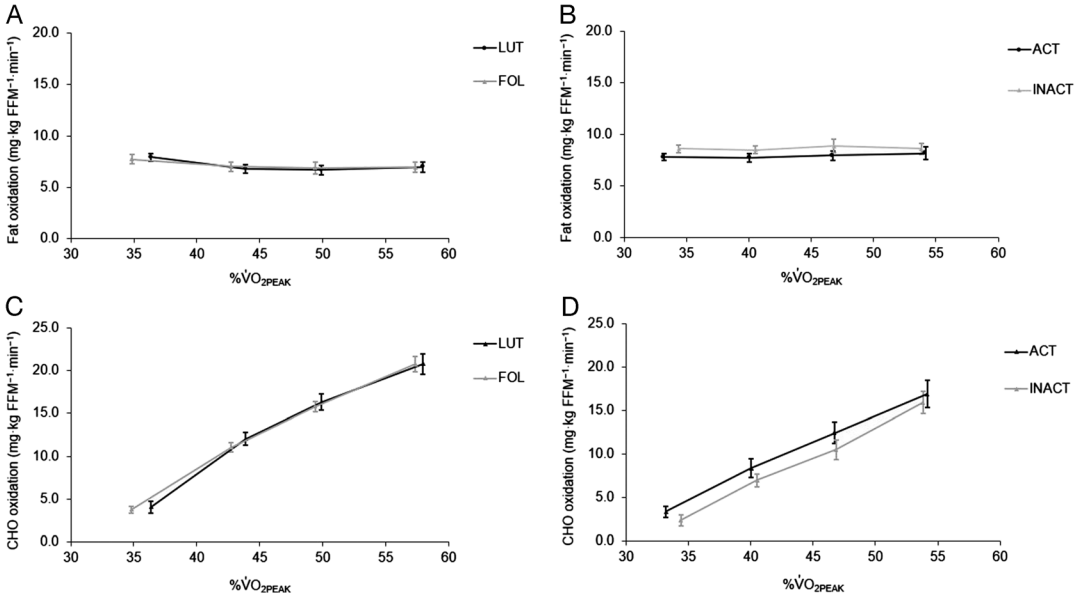

FIGURE 3—Relationships between intensity expressed as percentages of $\dot{V}O_{2peak}$ and substrate oxidation relative to FFM at test stages 1–4. Fat oxidation: (A) NoOC group, (B) COC group. Carbohydrate oxidation: (C) NoOC group, (D) COC group. Data are presented as mean ± SE.

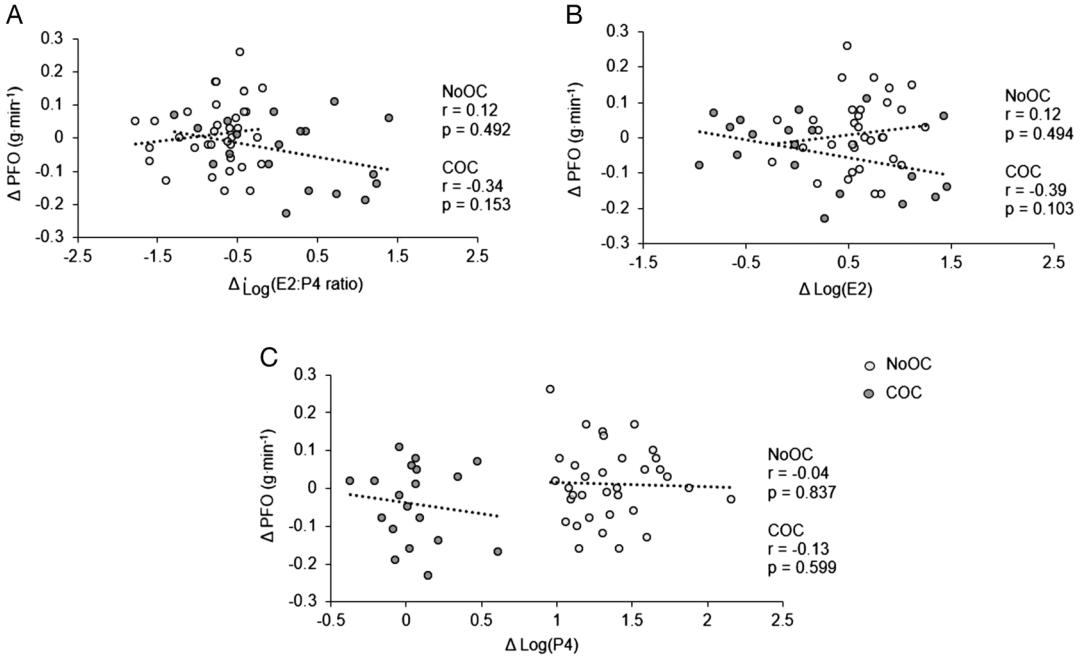

FIGURE 4—Associations between the changes in PFO and the changes in (A) log-transformed E2:P4 ratio, (B) log-transformed E2, (C) log-transformed P4. Δ = LUT–FOL or ACT–INACT. E2, estradiol; P4, progesterone.

CI = −0.04 to 0.07 g·min^{-1}, P = 0.601) and FAT_{MAX} was 3.9% (95% CI = −3.5% to 12.3%, P = 0.271) higher compared with the LUT.

We used multiple linear regression to examine between-group differences in PFO while adjusting for the main confounding variables, FFM and $\dot{V}O_{2peak}$. Detailed results are presented in Table 3. In FOL/INACT, PFO remained 0.07 g·min^{-1} (95% CI = 0.01 to 0.14 g·min^{-1}, P = 0.028) higher in the COC group compared with the NoOC group (Fig. 5). However, after excluding an outlier, the between-group difference was no longer statistically significant (B = 0.05 g·min^{-1}, 95% CI = −0.01 to 0.11 g·min^{-1}, P = 0.108). In LUT/ACT, the FFM- and $\dot{V}O_{2peak}$-adjusted PFO did not differ significantly between the groups.

Finally, the associations between FFM- and $\dot{V}O_{2peak}$-adjusted PFO residuals and FM are presented in Supplemental Figure 1 (Supplemental Digital Content 1, http://links.lww.com/MSS/D187). FM was a significant predictor of PFO in the NoOC group in the FOL (β = 0.66, P = 0.002), as well as in the entire sample in the FOL/INACT (β = 0.42, P = 0.002). No significant relationships were observed in the COC group during INACT, or LUT/ACT, either in the full sample or in subgroup analyses.

DISCUSSION

The present study investigated the influence of MC phases and COC phases on PFO and compared differences in substrate metabolism during graded submaximal treadmill exercise, performed in a fasted state, between NoOC and COC groups. Contrary to some previous studies that used protocols such as 10-min incremental treadmill test at submaximal intensities ranging from 35% to 75% of $\dot{V}O_{2MAX}$ (14), or single 1-h cycling protocols at fixed intensities of 60% (15) or 65% of $\dot{V}O_{2MAX}$ (16), our findings of no clear differences in substrate metabolism across the MC align with those of a recent systematic review and meta-analysis (23) as well as studies investigating PFO during

TABLE 3. Results of multiple linear regression analysis examining whether $\dot{V}O_{2peak}$, FFM, and grouping variable explain differences in PFO.

	FOL vs INACT				LUT vs ACT			
Independent variable	**B (95% CI)**	**β**	***P* Value**	**Adj. R^2**	***B* (95% CI)**	**β**	***P* Value**	**Adj. R^2**
A. Results before the sensitivity analysis								
	Dependent variable: PFO (*n* = 48)				**Dependent variable: PFO (*n* = 50)**			
$\dot{V}O_{2peak}$ (L·min^{-1})	0.069 (−0.094 to 0.233)	0.17	0.398	0.171	0.094 (−0.057 to 0.244)	0.25	0.216	0.061
FFM (kg)	0.003 (−0.005 to 0.011)	0.13	0.478		0.002 (−0.007 to 0.010)	0.37	0.711	
Group (COC = 1; NoOC = 2)	−0.072 (−0.138 to −0.006)	−0.32	**0.033**		−0.026 (−0.088 to 0.035)	−0.13	0.390	
B. Results after the sensitivity analysis								
	Dependent variable: PFO (*n* = 47)				**Dependent variable: PFO (*n* = 49)**			
$\dot{V}O_{2peak}$ (L·min^{-1})	0.095 (−0.048 to 0.239)	0.27	0.188	0.169	0.114 (−0.033 to 0.260)	0.31	0.125	0.055
FFM (kg)	0.002 (−0.005 to 0.009)	0.10	0.614		0.00 (−0.008 to 0.008)	0.01	0.968	
Group (COC = 1; NoOC = 2)	−0.048 (−0.107 to 0.011)	−0.24	0.108		−0.013 (−0.073 to 0.048)	−0.06	0.680	

Significant differences bolded ($P \leq 0.05$).
FOL, follicular phase; INACT, inactive phase; LUT, luteal phase; ACT, active phase; B, unstandardized coefficient; β, standardized coefficient; Adj. R^2, adjusted R^2; $\dot{V}O_{2peak}$, peak oxygen uptake.

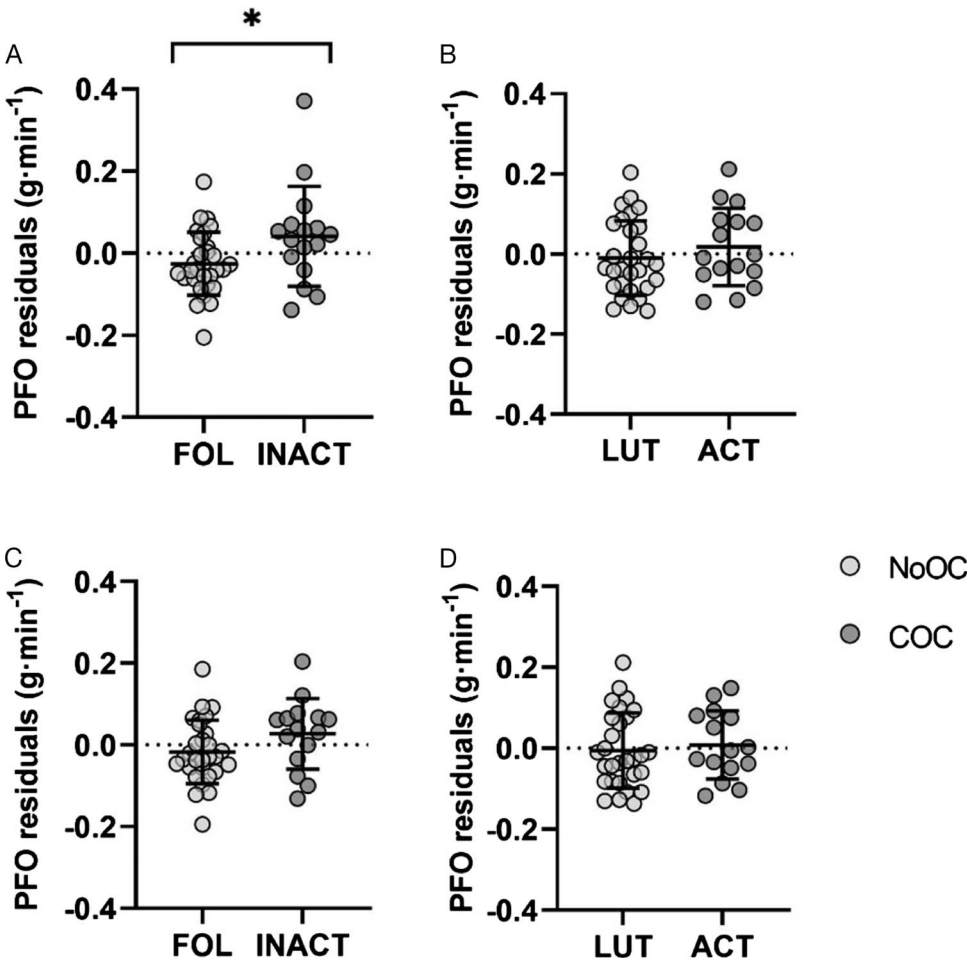

FIGURE 5—Comparison of $\dot{V}O_{2peak}$- and FFM-adjusted PFO at (A) FOL/INACT and (B) LUT/ACT before the sensitivity analysis, and at (C) FOL/INACT and (D) LUT/ACT after the sensitivity analysis. Residuals from the linear regression model, representing the difference between measured and predicted PFO based on $\dot{V}O_{2peak}$ and FFM, are used for visualization. Data are presented as means with SD and individual data points. *$P \leq 0.05$.

cycling (20,25). In the COC group, PFO remained relatively stable between phases.

We observed significantly higher PFO in the INACT of the COC group compared with the FOL of the NoOC group. However, the group difference was not robust against the removal of an outlier in the COC group. Notably, fluctuations in E2, P4, or the E2:P4 ratio were not associated with changes in PFO in either group. This suggests that the higher PFO observed in the COC group might be influenced by factors other than the endogenous hormones measured in this study, such as the potential metabolic effects of exogenous hormones (29).

Substrate metabolism during two phases of the MC or the COC. The ongoing debate regarding the influence of the MC on the substrate oxidation can be linked to several factors, including individual hormone profile variations, exercise protocols, and participants' training status (23). Hackney et al. (22) proposed that some studies may not detect phase-related fluctuations due to the minimal changes in E2 relative to P4 between phases, as P4 is suggested to counteract the lipolytic effects of E2 (9). Indeed, some research supports lower carbohydrate oxidation during 90 min of cycling at 65% of $\dot{V}O_{2peak}$ (47) or higher fat oxidation (22) during a 1-h running session at 65% of $\dot{V}O_{2MAX}$ in the LUT compared with the FOL has reported increases in E2 with relatively smaller increases in P4 (i.e., E2:P4 ratio change). However, this finding is not consistent. For instance, Vaiksaar et al. (19) reported a fourfold increase in E2 and a 17-fold increase in P4 from FOL to LUT but failed to detect MC phase effect on substrate oxidation during a 1-h rowing session at 70% of $\dot{V}O_{2MAX}$.

In our study, we observed a threefold increase in E2 and a 21-fold increase in P4 from early FOL to mid-LUT, suggesting that the relative change in E2 to P4 may not have been large enough to affect substrate metabolism. Conversely, Frandsen et al. (20), who found no differences in substrate metabolism during a cycling graded exercise test (3-min stages with 25 W increments) between MC phases, conducted measurements also in the late FOL, when E2 is highest relative to P4. They reported an approximately fourfold increase in E2 from mid-FOL to late FOL, whereas P4 levels remained unchanged between these phases. However, as these increases were calculated using the medians reported by the study, results are not fully comparable with our calculations using mean values. On the other hand, Hackney et al. (48) observed higher fat oxidation during 1 h of cycling at 70% of $\dot{V}O_{2MAX}$ at the ovulatory phase compared with the mid-FOL

with a 12-fold increase in E2 and a fivefold increase in P4, indicating a smaller change in the E2:P4 ratio compared with the results reported by Frandsen et al. (20). These discrepancies underscore the complexity of determining how the MC might affect substrate metabolism and support the need for further research that includes measurement of serum E2 and P4 to better understand the effect of E2:P4 ratio on substrate metabolism. It is important to note that studies cited used varying exercise modalities, including cycling, running, and rowing, with different intensities and durations. Optimally, comparisons should focus on protocols with greater methodological consistency.

Because the physiological effects of exogenous EE and progestin differ from those of endogenous hormones, the hypothesis regarding the E2:P4 ratio cannot be directly generalized to COC users (29). Moreover, we did not measure exogenous hormone concentrations in the present study. EE is suggested to have a stronger effect on hepatic metabolism than endogenous E2, whereas certain progestins may shift the fuel selection from carbohydrates toward fats by reducing the enzyme activity associated with glycolysis (29,30). Theoretically, the withdrawal of EE and progestin during the INACT should allow for a slight increase in endogenous E2 and P4 (12). In our study, average levels of endogenous E2 and P4 were lower in the INACT compared with ACT, likely due to the continued suppressive effect of exogenous hormones on the hypothalamic–pituitary–ovarian axis (49). We observed higher fat oxidation relative to FFM in stages 1 and 2 (30%–40%) in the INACT compared with the ACT, but difference was not statistically significant. Previous research on fat oxidation across COC phases generally indicates no clear differences between COC phases (27,33). Taken together, these findings suggest that variation in EE and progestin during the COC cycle has little or no effect on substrate metabolism.

To observe noticeable changes in fat oxidation, the exercise duration must be sufficient for lipolysis to reach its peak (50), particularly because E2 stimulates this process (51). Although the graded exercise protocols may be advantageous for detecting PFO by using multiple gradually increasing intensities, the short duration of the test (i.e., stage duration) could be a potential limiting factor in achieving maximal lipolysis (52). Chrzanowski-Smith et al. (38) investigated the validity of 4-min stages in the determination of PFO during cycling in untrained individuals and found substantial day-to-day variation, as well as poor surrogate validity to longer-duration stages testing. In the present study, fat oxidation was high at low intensity during the PFO test in both groups (Fig. 3). This effect might be amplified by fasting, as fatty acid availability is known to exceed the fat oxidation rate during exercise in the fasted state (53), as well as the relatively low cardiorespiratory fitness status of the participants. Previous studies have reported that FAT_{MAX} typically occurs below 50% $\dot{V}O_{2peak}$ in untrained participants in both graded cycle ergometer tests (45,54) and graded treadmill tests (55), which was observed in the NoOC group of this study. However, it is important to note that participants in these studies included both women and men of various ages, which differs from the population examined in our study.

In summary, we did not observe clear differences in substrate metabolism within the NoOC group, which does not necessarily imply the absence of such differences existing. Individual variability in hormonal fluctuations as well as methodological inconsistencies could contribute to the incongruency of results in the field (23). Moreover, the tendency for higher fat oxidation during INACT compared with ACT suggests that fluctuations in substrate oxidation might occur during a COC cycle. However, as research is limited and controversial, controlling for the COC phase may not be necessary in research investigating substrate metabolism during exercise.

Differences in PFO between NoOC and COC groups. We found higher PFO and FAT_{MAX} in the COC group compared with the NoOC group during both phases, with significant differences observed only at FOL/INACT. The higher PFO in the INACT was not explained by $\dot{V}O_{2peak}$ or FFM, but after excluding the participant with a notably higher PFO, the difference became smaller and was no longer statistically significant.

The more pronounced actions exerted by exogenous EE and progestin contained in COC on substrate metabolism compared with endogenous E2 and P4 may explain our observations (29). Another mechanism by which COC use may lead to higher fat oxidation is related to their tendency to elevate cortisol levels during submaximal exercise (56). Casazza et al. (56) found that after four months of triphasic COC use, including 0.035 mg EE and 0.18–0.215 mg norgestimate, women exhibited higher cortisol levels associated with increased triglyceride mobilization during moderate intensity exercise (45%–60% $\dot{V}O_{2peak}$). Nevertheless, this study did not find a significant change in fat oxidation despite the increased fat mobilization (56,57). The mismatch between these findings could be related to increased reutilization of mobilized fats (56,57).

On the other hand, Redman et al. (30) reported a shift from carbohydrate to fat utilization based on RER values during steady-state exercise performed at 75% of $\dot{V}O_{2peak}$ on a cycle ergometer in participants using COC with higher doses of progestins (1000 μg norethisterone) compared with those with lower doses (500 μg norethisterone), suggesting a potential glycogen-sparing effect, although the combined effect of EE could not be excluded. This is further corroborated by findings of Bemben et al. (26) (35 μg EE + 0.5–1.0 mg orethindrone or ethynodiol diacetate) and Williams et al. (27) (second-generation COC), who reported reduced carbohydrate oxidation during a 90 min treadmill test at 50% of $\dot{V}O_{2MAX}$ (26) or during two 8-min cycling bouts at 45% and 65% of $\dot{V}O_{2peak}$ (27) in women using COC compared with women not using COC. Our study included participants using second-, third-, and fourth-generation COC with varying doses of EE (20 to 30 μg) and progestin (30 μg to 3 mg). Our results align with those of Isacco et al. (25), who reported significantly higher PFO and FAT_{MAX} tested by graded cycling test including 6-min stages with increments of 10% of the maximal aerobic power in women using low-dose COC (20–30 μg EE + gestodene or levonorgestrel) compared with naturally menstruating women. However, the measurements conducted by Isacco et al. (25) were planned to occur between days 1 and 21 of ACT and between

days 18 and 22 of the MC, with no measurements conducted during FOL and INACT. Furthermore, the hormone levels of women not using COC reflected the early LUT instead of mid-LUT (25) and therefore are not completely comparable with ours. By contrast, an earlier study with a similar design, but using a different exercise protocol (45-min steady-state cycling at 65% of $\dot{V}O_{2MAX}$), found no differences in substrate metabolism between women using low-dose COC (20–30 μg EE + gestodene, drospirenone, or levonorgestrel) and those not using COC (58).

Overall, protocol variations likely contribute, to some extent, to these discrepancies. For instance, participant's nutritional status before the testing varied; i.e., some studies reported that testing was completed after an overnight fast (25,26), others a 4- to 6-h fast (27,30), and some allowed a standard breakfast (56,57) before assessment. Oosthuyse et al. (50) emphasized the importance of conducting measurements in a fasted state to obtain valid results, as substrate metabolism is greatly influenced by nutritional practices. The influence of habitual dietary intake on PFO is currently unclear, as some studies have indicated that carbohydrate and fat intake predict PFO to a small extent (59) and some have reported that it is not an important determinator of PFO (45). We could not investigate the contribution of dietary intake to the variance of PFO, as we did not standardize diet in the days preceding the PFO test due to the nature of our study. However, we otherwise ensured controlled conditions, including a 10-h fast, and that participants refrained from vigorous exercise 24-h before the test.

In this study, the body composition among participants varied substantially. Although most evidence proposes no significant differences in fat oxidation between overweight or obese and normal weight individuals (60), some studies indicate that people classified as obese may have higher fat oxidation during exercise due to greater intramuscular triglyceride availability and reduced muscle glycogen content (61,62). We found that FM was a significant predictor of PFO in the full sample in the FOL and INACT, as well as within the NoOC group in the FOL, even after controlling for FFM and $\dot{V}O_{2peak}$. These findings suggest that the influence of FM might warrant consideration in populations with highly variable body composition.

Enhanced reliance on fat during exercise can spare glycogen, potentially improving endurance performance (63), which may have important practical applications for women in sports. However, research exploring the influence of COC use on performance generally shows no drastic influence of COC on performance and emphasizes an individualized approach (28). In addition, the women investigated in the present study were recreationally active and had no competitive athletic goals. In this population, any marginal increase in fat oxidation and consequently, glycogen sparing, is unlikely to have meaningful benefits for performance during exercise, if not of a prolonged nature (50). Therefore, the physiological consequences of our findings remain unclear. Further research with larger samples and controlled conditions is necessary to elucidate the specific mechanisms and effects of different COC formulations on substrate metabolism.

Strengths and limitations. Our study has several strengths. We used a rigorously designed protocol, including the PFO test under consistent fasting conditions, and excluded participants with P4 levels below <16 nmol·L^{-1} at the LUT or with MC length aberrations. This strict inclusion criteria improves the validity of our data for eumenorrheic cycles (12). Additionally, our sample size in the NoOC group was larger than those in most previous studies, which increases the reliability of our findings.

However, our study also has some limitations. To accurately capture the peak increase in E2 relative to P4 during the MC, it is essential to measure hormone levels approximately a day preceding ovulation (12). Because of logistical constraints, regrettably, we could not measure participants during this critical phase. The participants in the COC group used a variety of COC containing progestins from different generations, potentially inducing varying effects of metabolism (64). To maintain sample size and statistical power, we did not subdivide participants by type of COC used. We relied on stoichiometric equations to calculate substrate metabolism from respiratory gases measured with indirect calorimetry. Although this method has some limitations, such as potential inaccuracies in gas exchange measurements and assumptions within the equations (42), it has been shown to be a valid method for assessing substrate oxidation during exercise when compared with the tracer methodology (65). However, we did not measure the contribution of protein to metabolism and instead assumed protein oxidation to be negligible.

Another limitation of the PFO test is the relatively short duration of its stages, as previous research has questioned the validity of 4-min test stages for accurately representing substrate use during prolonged exercise (38), where fat oxidation is maximally stimulated. Nevertheless, 4-min stages are sufficient for the achievement of steady-state gas exchange (38), which is essential for the accurate assessment of substrate metabolism using indirect calorimetry (66). Therefore, graded submaximal tests with 4-min stages are reasonably recommended to assess substrate oxidation in participants with low cardiorespiratory fitness (38). However, the participants in the present study reached PFO at lower intensities, which indicates that the starting intensity of PFO test conducted by researchers should be sufficiently low for recreationally active individuals.

Lastly, we measured participants for only one MC or COC cycle, which may limit our ability to detect the effects of hormonal fluctuations due to high day-to-day variability in PFO (40). Despite these limitations, our study provides valuable insights into the metabolic responses related to different hormonal environments. This contributes to the existing body of knowledge and enhances our understanding of hormonal influences within the MC or COC cycle on metabolism.

CONCLUSIONS

Substrate metabolism remained stable throughout both the MC and the COC cycle, but we found some evidence that COC users exhibited slightly higher PFO and FAT_{MAX} than naturally menstruating women (NoOC), particularly in the

INACT phase of the COC cycle. These findings suggest that exogenous EE and progestin may exert distinct effects on substrate metabolism compared with endogenous E2 and P4. Researchers and professionals in health and fitness, such as coaches and nutritionists, should be aware of how COC might affect substrate metabolism. However, generalized conclusions should be avoided due to the complexity of the factors involved.

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