Title: Menstrual cycle phase has no influence on performance-determining variables in endurance-trained athletes: the FENDURA project

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Abstract:

Female athletes frequently perceive performance changes throughout the menstrual cycle (MC). However, if and how the MC influences performance-determining variables remains unclear. **Purpose**: To investigate the effect of the MC and endogenous sex hormone concentrations on performance-determining variables in three distinct MC phases in endurance-trained females. **Methods**: Twenty-one eumenorrheic trained/highly trained endurance athletes completed a standardized test battery during the early follicular phase (EFP), ovulatory phase (OP), and mid-luteal phase (MLP) for either one (n=7) or two test cycles (n=14). MC phases were determined using calendar-based counting, urinary ovulation testing, and verified with serum hormone analysis. MCs were retrospectively classified as eumenorrheic or disturbed. Disturbed MCs were excluded from analysis. The test battery consisted of 4–6 x 5-min submaximal stages with stepwise speed increases, a 30-s all-out double-poling ski ergometer test, and a maximal incremental treadmill running test. **Results**: At a group level, there was no effect of MC phase or the serum concentrations of estrogen and progesterone on peak oxygen uptake ($\dot{V}O_{2peak}$), oxygen uptake at 4 mmol·L^{-1} blood lactate concentration, time-to-exhaustion, running economy, or mean 30-s power output (MPO_{30s}). Serum testosterone concentration was positively associated with MPO_{30s} ($p=0.016$). Changes in $\dot{V}O_{2peak}$ from EFP to MLP were inconsistent between individuals and across cycles. **Conclusions:** None of the measured performance-determining variables were influenced by MC phase or serum estrogen or progesterone concentrations. While some individual patterns could be observed, there was no indication that any single MC phase is consistently associated with improved or impaired $\dot{V}O_{2peak}$ on a group level.

Key Words: Oestrogen, Female Athletes, Maximal Oxygen Uptake, Progesterone, Running Economy, Sex Hormone

INTRODUCTION

Endurance performance is primarily determined by maximal oxygen uptake ($\dot{V}O_{2max}$), fractional utilization of $\dot{V}O_{2max}$, anaerobic capacity, and working economy or efficiency (1). Sex differences in many of these performance-determining variables are evident and well-documented (2, 3). However, the influence of the menstrual cycle (MC) and the associated hormonal fluctuations on performance-determining variables remains sparse and inconclusive (4). Understanding the effects of the MC is particularly relevant to highly trained women, given that small changes in performance-determining variables could be practically significant in determining performance outcomes. Additionally, the possible effect of the hormonal fluctuations on performance and performance-determining variables has been mentioned as a reason for the exclusion of female participants in sport science studies (5).

The eumenorrheic MC is a vital biological rhythm where endogenous sex hormones, mainly estrogen (E2) and progesterone (P4), fluctuate within a predictable 21–35-day cycle. Several hormonally distinct phases can be identified across a eumenorrheic MC, such as: the early follicular phase (EFP), indicated by the start of menstrual bleeding and characterized by low E2 and P4; the ovulatory phase (OP), within 36-h of a positive ovulation test when E2 is elevated and P4 remains low; and the mid-luteal phase (MLP), characterized by high E2 and P4. E2 and P4 modulate the MC, but also target several other physiological systems that may influence exercise performance (6, 7). Specifically, some studies found the hormonal fluctuations associated with the MC to influence arterial function (8), substrate metabolism (9, 10), neuromuscular function (11) and core body temperature (12). However, more recent reviews have suggested no effect (7, 13). The effects of E2 and P4 are dose-dependent and interrelated, and large individual differences have been observed in the magnitude of hormonal fluctuations throughout the MC (14, 15). Thus, it has been suggested

that the hormonal effects associated with MC phases may be regulated by the absolute and/or relative concentration of E2 to P4 (E:P ratio) (7). Accordingly, MC phase, or the related changes in E2, P4 or the E:P ratio throughout the cycle could influence performance-determining variables associated with endurance performance.

Previous studies indicate that 50–80% of athletes perceive their physical fitness and/or performance to be impaired in certain phases of the MC (16-18). However, most studies that have objectively assessed the effect of MC phase on endurance performance did not report any significant changes in performance metrics, such as time-to-exhaustion (TTE) (19-21) or maximal running speed (20, 22). Some studies have reported that the main performance-determining variables (i.e., $\dot{V}O_{2max}$, $\%\dot{V}O_{2max}$, anaerobic capacity, and running economy [RE]) are unaffected by MC phase (20, 21, 23-26). Others have observed small but significant changes in $\dot{V}O_{2max}$ (19, 23, 27), RE (26, 28), and peak power output (PPO) during short duration sprints (29). Unfortunately, there is little consistency within previous research as to the direction of change between phases. However, a recent meta-analysis indicated a trivial effect of MC phase on endurance-based outcomes and a trend towards a reduced performance in EFP compared to other phases (4).

The observed discrepancy in the results from studies investigating the effect of the MC can be potentially explained by differences in study design and methodology used. For example, McNulty et al. (4) reported that much of the available research was of “low” or “very low” quality. Identification and verification of MC phases is a critically important aspect of MC research to ensure accurate comparison, grouping of the hormonally-distinct phases, and the exclusion of participants with menstrual irregularities (30). However, previous studies have predominately utilized calendar-based counting to define MC phases rather than the recommended three-step MC phase verification method (i.e., calendar-based counting,

identification of ovulation, and verification of hormonal status) (31). Compared to sedentary women, physically active women exhibit a higher prevalence of subtle menstrual disturbances (i.e., anovulation or luteal phase defects), which largely influence the hormonal profile and are not detectable if only using a calendar-based counting method (30, 32, 33). Thus, in the absence of biological confirmation of MC-phases, the ability to conclusively evaluate the potential effects of MC-phase or sex hormones on endurance performance or performance-determining variables is limited.

Although several previous studies have examined the effect of MC phase on different performance-determining variables (4), additional high-quality research is necessary to understand how the underlying hormonal fluctuations influence the physiological components of endurance performance. Therefore, the primary aim of the current study was to investigate the effect of MC phase on performance-determining variables in endurance-trained athletes using gold-standard methodological procedures for MC phase determination. The secondary aim was to investigate the association between endogenous sex hormone concentrations (i.e., E2, P4, testosterone (T) and E:P ratio) and performance-determining variables.

METHODS

Experimental Design

The study was executed in two parts: the lead-in and the test-period (Figure 1). During the lead-in period, two complete MCs were systematically tracked using calendar-based counting and at-home urinary ovulation prediction tests. In addition, approximately two weeks before the test period, participants visited the laboratory for a familiarization session of the test battery. During the test period, participants completed a test battery in each of the three MC phases (EFP, OP and MLP) for up to two test cycles. Half of the participants were block randomized to start in either EFP, OP or MLP, and the other half started in the EFP. Each test cycle included

three consecutive tests regardless of start phase. All lab-based testing (including the familiarization session) occurred within a time frame of 3–5 consecutive MCs. Throughout the entire study period, participants were instructed to maintain a stable training load and self-recorded all training sessions on a day-to-day basis using one of two identical online training diaries: The Norwegian Top Sport Center (Olympiatoppen) online training diary (olt-dagbok.no), or BESTR.no (Oslo, Norway).

The current study was part of the Female Endurance Athlete (FENDURA) project, as previously described (34). This study was conducted at five different testing locations across Norway, with the same laboratory equipment and identical testing procedures. The study was evaluated by the Regional Committee for Medical and Health Research Ethics (REK, Project-ID: 230505) and approved by the Norwegian Centre for Research Data (NSD, Project-ID: 955558). All participants received oral and written information about the study procedures and provided their written informed consent to participate. Participants were told that they could withdraw from the study at any time without giving a reason for doing so.

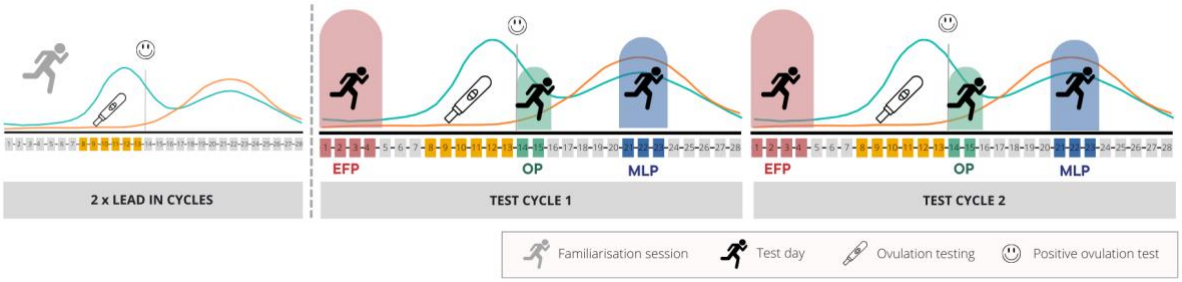


Figure 1. Illustration of study design over a hypothetical 28-day cycle. NB: 'Lead in cycles' refers to the two menstrual cycles between enrollment and the onset of the study period. Test cycles refer to the two menstrual cycles where the standardized testing took place. Running icon illustrates possible test days in the laboratory for each phase; EFP: early follicular phase (red days); OP: ovulatory phase (green days); and MLP: mid-luteal phase (blue days). Urinary testing (yellow days) was conducted from day 8 until a positive ovulation result. The colored lines represent theoretical sex hormone concertation across the menstrual cycle, with orange line: progesterone; blue line: estrogen.

Participants

Naturally menstruating, endurance-trained female athletes were recruited through Norwegian sporting organizations, personal connections, and social media. During the pre-screening process, participants completed an online questionnaire regarding the inclusion/exclusion criteria. Participants were invited to enroll in the study if they fulfilled the following pre-screening criteria: 1) reported a regular MC cycle length (between 21 and 35 days) for the last six months; 2) not using hormonal contraceptives for at least three months prior to the onset of the study; 3) aged 17–40 years; 4) engaged in systematic training in an endurance sport for at least the past three years; 5) completing a minimum of five endurance training sessions per week. Participants were ineligible to participate if there was evidence of: 1) injury or illness that prevented them from training regularly; 2) a clinically diagnosed menstrual disorder (e.g., polycystic ovarian syndrome or amenorrhea), 3) pregnancy; or, 4) having given birth within the 12 months prior to the start of the study.

Of the 71 participants that enrolled in the project, 32 completed the study period and 21 were included in the final analyses (see Figure 2). Participants were classified as trained (tier 2, n = 11) or highly trained (tier 3, n = 10) endurance athletes (35).

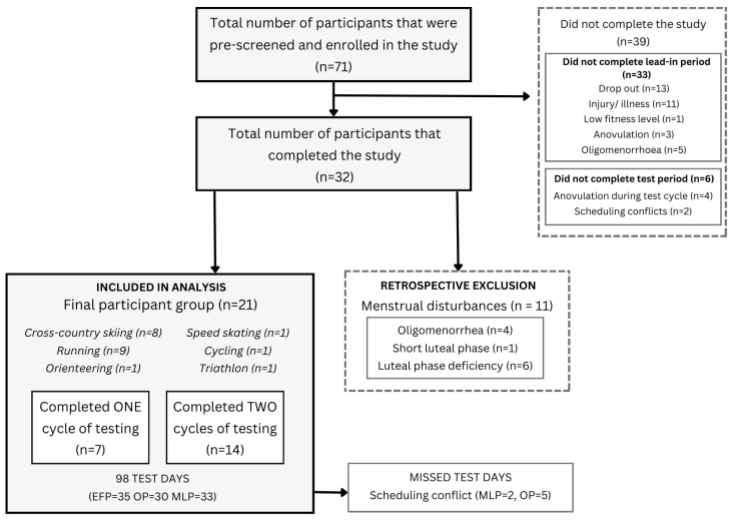


Figure 2. Flow chart of participant inclusion.

Menstrual Cycle Phase Determination

MC phases were determined using the "three-step method" which has previously been described in detail by Schaumberg et al. (31). Participants indicated their first day of menses in their training diary (step one). Subsequently, they used Clearblue Digital Ovulation kits (SPD Swiss Precision Diagnostics GmbH, Geneva, Switzerland) each morning starting from day eight until a positive result from the ovulation test, or until the first day of menses in the following MC (step two). The day of ovulation was considered as the day on which a positive urinary ovulation test result was detected. MC length was defined as the number of days from the start of menses in one MC to the day preceding the start of menses in the subsequent MC. Luteal phase length was defined as the number of days from the day after ovulation, up to the day preceding menses.

During the test period, participants visited the laboratory for one test day during each of three MC phases: EFP (day one to day four of the MC), ovulatory phase (OP) (within 36 hours of a positive ovulation test), and mid-luteal phase (MLP) (seven to nine days following the day of ovulation). Participants provided a fasted blood sample at the start of each test day, which was retrospectively analyzed for serum hormone concentration (step three). All MCs were retrospectively classified as eumenorrheic or having a menstrual disturbance.

Menstrual disturbances were defined as: a) oligomenorrhea, i.e., MC length greater than 35 days, but less than 90 days (36); b) anovulation, i.e., no ovulation detected by the urinary ovulation prediction test (36); c) short luteal phase, i.e., luteal phase shorter than 10 days; (32) and d) luteal phase deficiency, i.e., P4 concentration < 16 $nmol \cdot L^{-1}$ in MLP (30). Participants presenting with oligomenorrhea and/or repeated anovulatory cycles (i.e., more than one anovulatory cycle) during the lead-in period did not progress to the test period. MCs identified with one of the aforementioned menstrual disturbances during the test period were

retrospectively excluded from the analysis. All remaining MCs were classified as eumenorrheic (36) and included in the final analysis.

Test protocols

Familiarization Session

The familiarization test protocol was identical to the standardized test battery (described hereafter) except for the absence of blood sampling/breakfast procedures and the individualized intensity of the stages of the submaximal test (Figure 3). Data from this session were used to individually optimize the intensity of the stages of the submaximal test during the test period.

Test Day Procedures

Participants were instructed to prepare for each test session as they would for a competition, i.e., ensure optimal sleep, euhydration, and avoid high-intensity training within the preceding 24-h. They were also instructed to record their nutritional intake for the 24-h before arriving at the laboratory prior to the first test day and replicate this dietary intake before each subsequent test day. Participants were reminded of these procedures prior to each test day.

Participants arrived at the laboratory in a fasted state between 6 and 10 a.m. Arrival time was standardized for each individual (±1 h) across test days based on their preferred schedule. A venous blood sample was drawn from an antecubital venipuncture and processed by a certified technician. Participants were then provided with a standardized breakfast that aligned with pre-sporting event nutritional guidelines (i.e., 2 g carbohydrate per kg body mass) (37). The breakfast was consumed at the start of a 2-hour break, ensuring sufficient time to eat and digest. Test day procedures are illustrated in Figure 3.

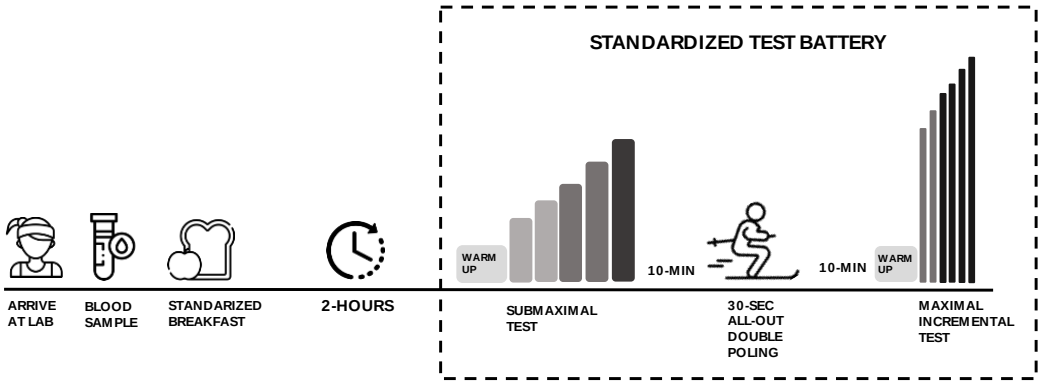


Figure 3. Illustration of test day

Warmup

When the participants returned to the laboratory, their height and body mass were recorded. Prior to the submaximal test the participants completed a 5-min warmup while running on a treadmill (Woodway PPS Med 55, Waukesha, Wisconsin, USA) at an incline of 10.5%. The warmup speed was set at approximately 55% of the velocity attained at $\dot{V}O_{2peak}$ from the familiarization session.

Submaximal Test

The submaximal test was performed as treadmill running with a 10.5% incline and consisted of 4–6 x 5-min stages of running with stepwise increases in speed and 1-min passive recovery between stages. The stage speeds were set to 65, 70, 75, 80, 85 and 90% of the velocity at $\dot{V}O_{2peak}$ for each individual using data from the familiarization session and kept the same throughout the study period. During the final 2 min of each stage, participants breathed into a 2-way breathing valve (2730 series, Hans Rudolph Inc, Kansas City, MO, USA), which was connected to a metabolic-gas analyzer in mixing-chamber mode (Vyntus CPX, Vyaire medical GMBH, Höchberg Germany). Respiratory data were recorded in 5-s increments, and the average of the final minute per stage was used for subsequent analysis. Between each stage,

the participant's rating of perceived exertion (RPE; 6–20 scale) (38) was recorded, and a capillary blood sample was collected from the fingertip and analyzed for baseline blood lactate concentration ($[BLa^-]$) on a lactate analyzer (BIOSEN C-Line GP+, EKF Diagnostics for life, Leipzig, Germany). The test was terminated upon either a $[BLa^-] \geq 4$ mmol·L^{-1} or an RPE >17, whichever came first. Data from the submaximal test was used to calculate running velocity, $\%\dot{V}O_{2peak}$, HR and RPE at the onset of blood lactate accumulation (OBLA, OBLA = 4 mmol·L^{-1} $[BLa^-]$) through interpolation (39). Any test in which the participant did not reach a minimum $[BLa^-]$ of 4 mmol·L^{-1} was excluded from the analysis of OBLA data (26 tests were excluded). RE was determined as $\dot{V}O_2$ in mL·kg^{-1}·km^{-1} at the individual speed closest to 80% of velocity at $\dot{V}O_{2peak}$ where RER was < 1.0 (40).

30-s All-out Double Poling

Ten minutes after the submaximal test, each participant performed a 30-s all-out double-poling test on a ski ergometer (SkiErg PM5; Concept 2 Inc., Morrisville, Vermont, USA). A self-selected foot position was recorded and used during subsequent tests. A 4-min low-intensity warmup was performed with a standardized double-poling technique. After the warmup, the participant came to a full stop in a starting position, with the upper arm parallel to the floor. Participants were counted down from "3" and instructed to explosively pull down on the handles and complete a 30-s all-out test. The resistance on the ski ergometer was set to zero throughout both the warmup and sprint. Investigators provided verbal encouragement throughout the test. RPE was collected immediately after the test and defined as RPE_{30s}. Mean PO (MPO_{30s}) and PPO were determined as the mean PO and peak PO during the 30-s test, respectively. This test is commonly used in Nordic sports to assess and measure maximal upper body anaerobic power and correlates well with performance outcomes in cross-country skiing events (41).

Maximal Incremental Test

After a 10-min break, the participant returned to the treadmill for a 5-min warmup at a speed of 6.0 $km \cdot h^{-1}$ and an incline of 10.5%. The test started at a speed of 7.0 $km \cdot h^{-1}$ and increased by 0.8 $km \cdot h^{-1}$ every minute until volitional exhaustion, or the participant refused to increase the speed further. Verbal encouragement was provided throughout the test. Respiratory data were collected throughout the test using the Vyntus metabolic-gas analyzer, as previously described. At the end of the test, RPE was recorded and defined as RPE_{peak}. Capillary blood samples were collected and analyzed for $[BLa^-]$ immediately following, and 3 min after the end of the test, from which the highest $[BLa^-]$ was defined as $[BLa^-]_{peak}$. $\dot{V}O_{2peak}$ and HR_{peak} were defined as the highest average 30-s $\dot{V}O_2$ or HR measurement using a moving average filter. TTE was defined as the duration of the maximal incremental test in seconds.

Blood sampling procedures and analysis

A venous blood sample was obtained from an antecubital venipuncture after an overnight fast. Blood samples were collected in serum separator tubes (Vacutainer SST 8.5 mL, BD, Franklin Lakes, NJ, United States) and left to clot for 30 min before centrifugation at 4200 rpm for 10 minutes. The serum was pipetted to a 5 ml Sarstedt tube and stored frozen at -80°C until analysis. The samples were analyzed by standard clinical procedures at the University Hospital of Northern Norway, Tromsø, Norway, accredited according to ISO/IEC 15189. The serum was analyzed for E2, P4, follicle–stimulating hormone (FSH), luteinizing hormone (LH), and T using liquid chromatography-tandem mass spectrometry. The method was validated and found to be linear from 0.03 to at least 20 nM for E2 with a squared correlation coefficient (r2) > 0.995, from 0.3 to at least 130 nM for P4 and from 0.1 to at least 130 nM for T (r2 > 0.995). Intraday precision values were evaluated by assaying three samples (low, medium, and high concentration) six times on the same day. The CVs for all analytes were < 6.5% for all three levels. All the quality controls were found to be well within acceptable limits. E:P ratio was calculated as E2 ($pmol \cdot L^{-1}$) divided by P4 ($nmol \cdot L^{-1}$) for each MC phase.

Consistency of $\dot{V}O_{2peak}$ Between Cycles

The change in $\dot{V}O_{2peak}$ from EFP to MLP was described for 12 participants with threshold-based classification over two cycles of testing (42, 43). A two-way threshold of 3% was set in accordance with the known measurement error for gas exchange variables as stated by the manufacturer (Vyntus CPX, Vyaire medical GMBH, Höchberg Germany). Each cycle was classified as; EFP positive (higher $\dot{V}O_{2peak}$ in EFP compared to MLP), MLP positive (higher $\dot{V}O_{2peak}$ in MLP compared to EFP), or no change (change in $\dot{V}O_{2peak}$ from EFP—MLP was within the threshold of measurement error). Participants were classified as having a consistent/inconsistent response by comparing the classification in cycle 1 and cycle 2.

Statistical Analysis

Sample size was calculated in G*power (44) assuming a medium effect size of 0.25 (45) (α = 0.05, power = 0.80, number of measurements = 6, correlation among repeated measurements = 0.5, non-sphericity correction = 1.0), which resulted in a sample size of 19 participants. To account for drop outs and a post hoc exclusion rate of ~40% (30), new participants were added until 32 participants had completed the study period.

Data were analyzed using linear mixed effects regression. The association between the dependent variables (e.g., TTE, etc.) and MC phase (fixed effect) were modelled with a random intercept for participant, with MC nested within participant. The alpha level was set at 5%. Post-hoc pairwise tests were corrected for multiple comparisons with the Tukey method. RPE was considered a continuous variable and analyzed as such. For the secondary aim, the relationships between sex hormones (main determinants) and the dependent variables were investigated using random intercept models with MC phase nested within participant. Unless otherwise stated summary data are presented as estimated marginal means and 95% confidence interval (CI) from the regression models. Mean difference (MD) between phases is presented when a main effect of MC phase was observed. Visual inspection of model residuals did not reveal obvious deviations from normality. All statistical analyses were performed using R in the RStudio environment (46), with the packages “lme4” (version 1.1-29) (47) “emmeans” (version 1.8.4-1)(48) and “ggplot2” (version 3.3.6) (49).

RESULTS

Menstrual cycle characteristics

A total of 116 unique MCs were recorded during the test-period. Menstrual disturbances were observed in 36% of the recorded cycles during the test-period and the associated tests were excluded from further analysis. EFP testing took place within day 1 to day 4 from the onset of menstruation (mean: day 3 ± 1), OP testing was always within 36 hours of a positive ovulation test (mean: day 16 ± 3), and MLP testing was within 7–9 days after a positive ovulation test result (mean: day 23 ± 3). Participant characteristics of the final group are shown in Table 1.

The sex hormone concentrations measured for each phase were reflective of eumenorrheic MCs (Table 2). There was a significant main effect of phase on all measured hormones. The average day of ovulation was 15.4 ± 2.8, with an inter-individual range between day 9 and 21. The average length of the luteal phase was 13.5 ± 1 days, with an inter-individual variability between 10 and 17 days in length.

Table 1. Characteristics of final participant group (n=21)

VARIABLE	MEAN ± SD
Age (years)	27 ± 7
Body mass (kg)	61 ± 6
Body height (cm)	167 ± 7
Peak oxygen uptake ($mL \cdot kg^{-1} \cdot min^{-1}$)	53.8 ± 5.6
Weekly training hours ($h \cdot week^{-1}$)	7.6 ± 3.2
Menstrual cycle length (days)	28.8 ± 3.0

TABLE 2. Serum concentrations of hormones and estrogen-to-progesterone ratio in three menstrual cycle phases (n=21)

HORMONE	EFP	OP	MLP
N	35	30	33
E2 (pmol·L^{-1})	127.0 (92.5-175.5)	319.5 (241.2-527.2)*	590.0 (457.0-787.0) #§
P4 (nmol·L^{-1})	0.68 (0.49-0.83)	3.52 (2.67-4.57)*	29.45 (26.81 – 40.05) #§
LH (IU·L^{-1})	6.8 (5.9-8.0)	13.5 (10.65-21.57)*	4.8 (3.0-7.0)§
FSH (IU·L^{-1})	7.3 (6.0-8.8)	7.8 (6.1-10.7)	3.0 (2.3-4.1)#
T (nmol·L^{-1})	0.73 (0.61-0.95)	1.05 (0.81-1.33)*	0.81 (0.70-0.95) §
E:P ratio	206.0 (133.3-398.2)	87.3 (53.6-196.0)	17.5 (13.3-23.3)#

Values are presented as medians and interquartile ranges.
* significant difference between EFP and OP
# significant difference between EFP and MLP
§ significant difference between OP and MLP
EFP: Early follicular phase; OP: Ovulatory phase; MLP: Mid-luteal phase. N: number of samples included in analysis; E2: estradiol; P4: progesterone; LH: luteinizing hormone; FSH: follicle stimulating hormone. T: testosterone. E:P ratio: estrogen to progesterone ratio.

Associations between sex hormones and performance-determining variables

There was no significant association between serum E2, P4 or E:P ratio (measured in the various MC phases) and any of the main determinants of endurance performance (Table 4). Circulating T was positively associated with both MPO_{30s} and PPO_{30s} ($p=0.016$, $p=0.002$, respectively).

Consistency of response between cycle 1 and cycle 2

Fourteen participants completed two cycles of testing. Two participants missed an MLP test due to scheduling conflicts and thus paired EFP—MLP $\dot{V}O_{2peak}$ data from 12 participants over two cycles was observed. Two participants were consistently EFP-positive, two were consistently MLP-positive, and two consistently no-change. Six participants had inconsistent classification between cycle 1 and cycle 2 (See Supplementary Figure 1).

304 **TABLE 3.** Performance-determining variables during the early follicular phase (EFP), ovulatory phase (O) and mid-luteal phase (MLP)

	EFP		OP		MLP		EFFECT OF PHASE	Mean within participant CV
VARIABLE	**MEAN**	**95% CI**	**MEAN**	**95% CI**	**MEAN**	**95% CI**	***P***	**%**
Body mass (kg)	61.4	58.7–64.1	61.5	58.9–64.0	61.2	58.7–63.8	0.170	1.1
Aerobic Performance								
Maximal Incremental Test								
TTE (s)	374	349–400	376	350–403	365	340–391	0.227	7.3
$\dot{V}O_{2peak}$ (mL·min^{-1})	3305	3170–3440	3336	3199–3473	3287	3152–3423	0.362	3.6
$\dot{V}O_{2peak}$ (mL·kg^{-1}·min^{-1})	54.2	52.0–56.3	54.7	52.5–56.9	54.2	52.0–56.4	0.527	3.5
HR_{peak} (beats·min^{-1})	188	183–192	188	184–193	187	183–192	0.237	1.2
BLa^{-}_{peak} (mmol·L^{-1})	8.9	7.9–10.0	9.0	7.9–10.0	8.2	7.2–9.3	0.057	15.7
RPE_{peak}	19.0	18.6–19.3	18.9	18.5–19.3	18.9	18.6–19.3	0.855	2.9
Onset of Blood Lactate Accumulation (4 mmol·L^{-1})								
Velocity (km·hr^{-1})	8.0	7.6–8.5	8.1	7.7–8.5	8.1	7.7–8.6	0.296	2.9
$\dot{V}O_2$ (%)	86.1	84.2–88.2	85.2	82.9–87.4	86.5	84.5–88.5	0.368	3.8
HR (beats·min^{-1})	176	171–181	177	172–182	178	173–183	0.142	1.5
RPE	15.6	14.9–16.2	15.1	14.5–15.8	15.2	14.6–15.8	0.109	4.9
Running Economy								
RE (mL·km^{-1})	21490	20296–22785	21329	20030–22628	21250	19954–22547	0.420	3.1
RE (mL·kg^{-1}·km^{-1})	350	342–358	347	339–356	348	340–356	0.659	3.0
Anaerobic Performance								
30-s All-out Double-Poling								
MPO_{30s} (W)	181	167–196	186	171–201	180	165–195	0.081	4.6
PPO_{30s} (W)	250	210–290	266	226–307	248	207–288	0.283	12.1
RPE_{30s}	16.6	16.0–17.3	16.9	16.2–17.5	17.2[#]	16.6–17.8	**0.043**	4.9

Values are presented as estimated marginal means; and 95% CI, 95% confidence interval.

[#] significant difference between EFP and MLP

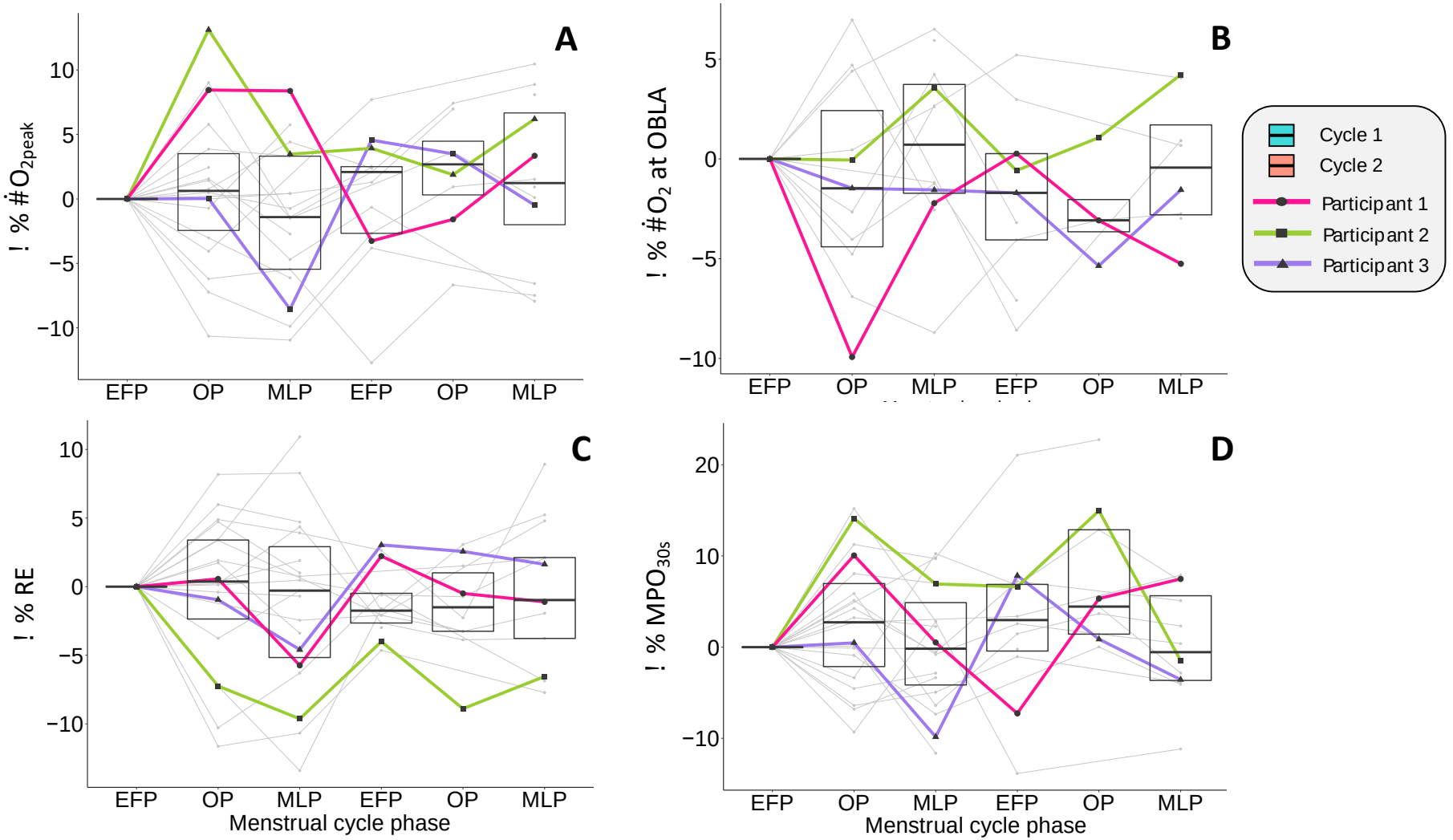
TTE, time-to-exhaustion; $VO_{2peak,}$ peak oxygen uptake; HR, heart rate; BLa^{-}_{peak}, peak blood lactate; RPE, rating of perceived exertion (6-20 Borg Scale); RE, running economy; MPO_{30s}, mean power output during 30-s all-out double-poling; PPO_{30s}, peak power output during 30-s all-out double-poling. Merged data from 21 participants; 7 participants completed one cycle of testing, and 14 participants completed two cycles of testing.

TABLE 4. The association between hormonal concentrations and performance-determining variables

	Estrogen			Progesterone			Testosterone			E:P Ratio		
VARIABLE	**EST.**	**95% CI**	***P***	**EST.**	**95% CI**	***P***	**EST.**	**95% CI**	***P***	**EST.**	**95% CI**	***P***
Aerobic Performance												
Maximal Incremental Test												
TTE (s)	-0.01	-0.03 – 0.02	0.555	0.19	-0.59 – 0.21	0.341	16.69	-12.39 – 45.77	0.257	0.01	-0.02 – 0.05	0.372
$\dot{V}O_{2peak}$ (mL·min^{-1})	-0.08	-0.19 – 0.04	0.181	-1.05	-2.93 – 0.83	0.271	38.64	-104.18 –181.47	0.592	0.02	-0.13 – 0.18	0.768
$\dot{V}O_{2peak}$ (mL·kg^{-1}·min^{-1})	-6.5e-4	-2.5e-3 – 1.2e-3	0.480	-0.01	-0.04 – 0.02	0.575	0.95	-1.36 – 3.25	0.416	-4.8e-4	-3.0e-3 – 2.1e-3	0.708
HR_{peak} (beats·min^{-1})	7.1e-4	-1.5e-3 – 2.9e-3	0.522	-0.02	-0.05 – 0.02	0.326	1.41	-1.48 – 4.30	0.334	1.2e-4	-2.8e-3 – 3.0e-3	0.933
BLa^{-}_{peak} (mmol·L^{-1})	-1.2e-4	-1.5e-3 – 1.2e-3	0.855	-0.02	-0.04 – 0.00	0.128	0.98	-0.46 – 2.41	0.179	1.7e-3	1.0e-4 – 3.3e-3	**0.038**
RPE_{peak}	1.0e-5	-4.8e-4 – 5.1e-4	0.954	2.0e-3	-0.01 – 0.01	0.540	0.07	-0.50 – 0.63	0.812	2.0e-4	-4.6e-4 – 8.6e-4	0.552
Onset of Blood Lactate Accumulation (4 mmol·L^{-1})												
Velocity (km·hr^{-1})	1.4e-4	-1.2e-4 – 4.0e-4	0.281	2.7e-3	-1.3e-3 – 6.7e-3	0.184	-0.02	-0.36 – 0.32	0.916	-2.3e-4	-5.3e-4 – 8.0e-5	0.139
$\dot{V}O_2$ (%)	1.7e-3	-1.4e-3 – 4.6e-3	0.289	0.03	-0.02 – 0.08	0.295	-2.08	-5.37 – 1.21	0.211	1.8e-4	-3.5e-3 – 3.9e-3	0.923
HR (beats·min^{-1})	0.03	-0.03 – 0.09	0.360	3.71	-1.05 – 8.47	0.124	-4.3e-3	-8.5e-3 – 4.0e-5	0.052	1.9e-3	-2.2e-3 – 5.9e-3	0.052
RPE	-5.9e-4	-1.4e-3 – 1,8e-4	0.133	-0.01	-0.02 – 0.01	0.281	-0.36	-1.24 – 0.51	0.412	1.1e-4	-8.1e-4 – 1.0e-3	0.814
Running Economy												
RE (mL·kg^{-1}·km^{-1})	-5.1e-3	-1.5e-2 – 4.9e-3	0.314	-0.07	-0.24 – 0.10	0.434	-8.04	-19.42 – 3.34	0.164	1.6e-3	-1.1e-2 – 1.5e-2	0.806
Anaerobic Performance												
30-s All-out Double-Poling												
MPO_{30s} (W)	-1.1e-3	-9.3e-3 – 7.0e-3	0.783	-0.10	-0.23 – 0.03	0.140	12.79	2.45 – 23.13	**0.016**	7.0e-3	-3.5e-3 – 1.8e-2	0.189
PPO_{30s} (W)	0.02	-0.01 – 0.06	0.198	-0.12	-0.71 – 0.47	0.689	62.74	23.51 – 101.97	**0.002**	4.2e-3	-0.04 – 0.05	0.855
RPE_{30s}	4.5e-4	-4.0e-4 – 1.3e-3	0.293	1.4e-2	1.3e-3 – 2.8e-2	**0.032**	-0.04	-0.98 – 0.90	0.929	-1.4e-3	-2.4e-3 – -4.4e-4	**0.005**

Values are presented as effect estimates, Est.; 95% confidence interval, 95% CI, P-value

TTE, time-to-exhaustion; VO_{2peak}, peak oxygen uptake; HR, heart rate; BLa^{-}_{peak}, peak blood lactate; RE, running economy; RPE, rating of perceived exertion (6-20 Borg Scale); MPO_{30s}, mean power output during 30-s all-out double-poling on ski ergometer; PPO_{30s}, peak power output during 30-s all-out double-poling on ski ergometer.


A
B
C
D
! % $\dot{V}O_{2peak}$
! % $\dot{V}O_2$ at OBLA
! % RE
! % MPO_{30s}
EFP
OP
MLP
Menstrual cycle phase
Cycle 1
Cycle 2
Participant 1
Participant 2
Participant 3

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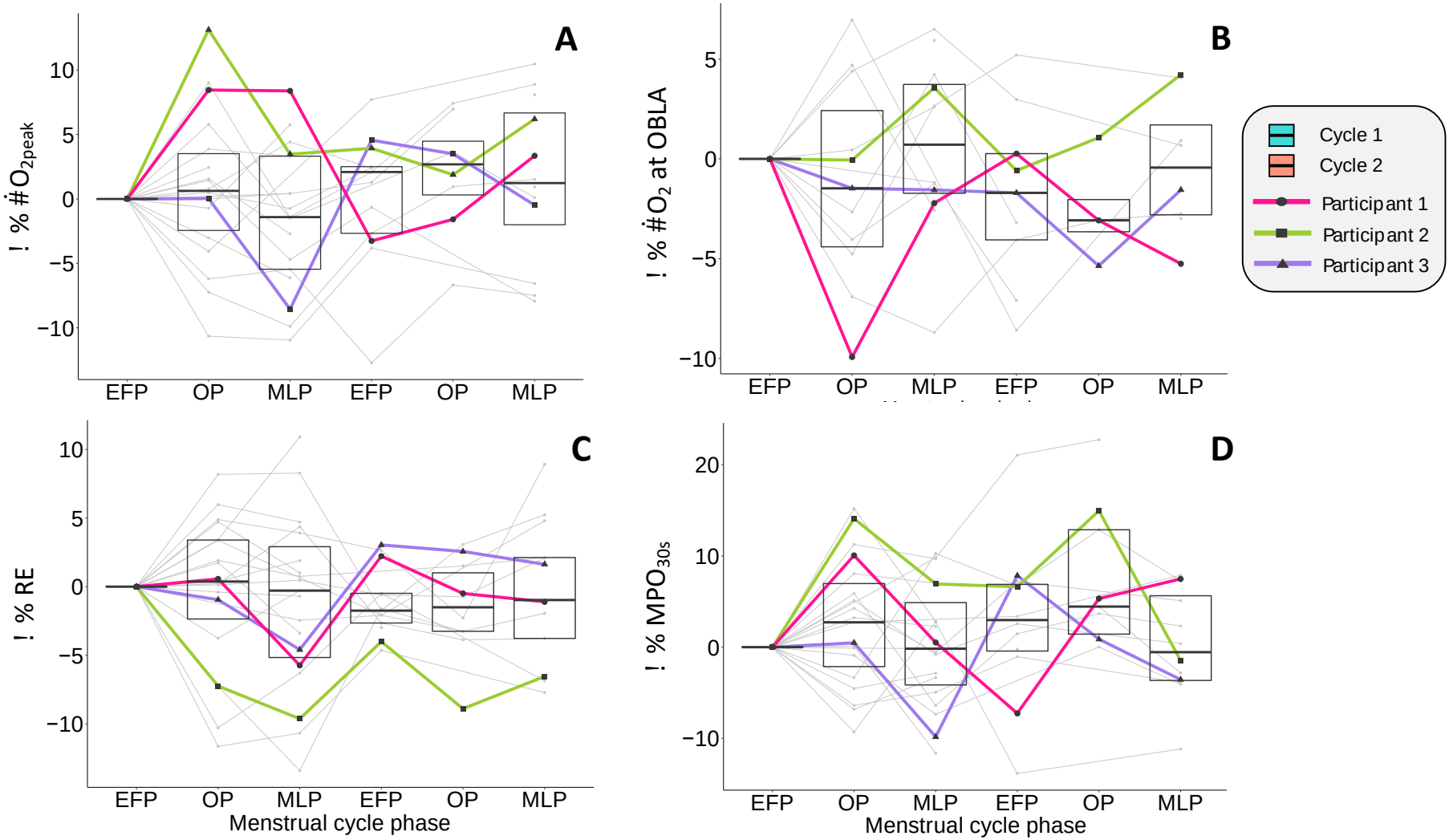

310

311 **Figure 4.** Percent change in performance-determining variables in different phases of the menstrual cycle over two cycles of testing. A) $\dot{V}O_{2peak}$, peak oxygen uptake ($mL \cdot kg^{-1} \cdot min^{-1}$); B) Percent
312 VO_{2peak} at OBLA; C) RE, running economy ($mL \cdot kg^{-1} \cdot km^{-1}$) D); MPO_{30s}, Mean power output during 30-s double-poling (W). Solid grey lines represent individual data. Box present median and
313 interquartile ranges. EFP, early follicular phase; OP, ovulatory phase; MLP, mid-luteal phase.

DISCUSSION

The main finding of the current study was that the performance-determining variables, such as $\dot{V}O_{2peak}$, $\%\dot{V}O_{2peak}$ at OBLA, RE and MPO_{30s}, did not significantly change between MC phases (EFP, OP, MLP). Coinciding with this observation, no significant associations between circulating sex hormones or the E:P ratio and the performance-determining variables were found. However, there were positive associations between T and PPO_{30s} and MPO_{30s}. The EFP–MLP changes in VO_{2peak} indicated between- and within-individual inconsistency between cycles.

Influence of MC phase on performance-determining variables

The finding that $\dot{V}O_{2peak}$ remained stable across MC phases and the lack of an association between $\dot{V}O_{2peak}$ and serum hormone concentrations or E:P ratio appears to be consistent with the majority of previous literature (20, 21, 26), although small improvements in absolute $\dot{V}O_{2max}$ (~2%) have been found in the EFP compared to MLP as well (19). In the current cohort, inter-individual variation was observed across the MC. For some athletes, $\dot{V}O_{2peak}$ stayed relatively stable (i.e., fluctuated <3%) throughout the MC, while for others, it varied by approximately ±10% across phases. Similar variation can be observed in the individual data presented by Taipale et al. (20) and Gordon et al. (23). Beyond the MC, differences between repeated measurements can be attributed to a number of factors, including machine/tester error or biological variation (50). Normal day-to-day variation for $\dot{V}O_{2max}$ measurements is reported to be around 3-5% (50), consistent with the present findings (CV=3.5%). Taken together, there is limited evidence to support the notion that $\dot{V}O_{2max}$ measurements are susceptible to phase-based or hormonal fluctuations.

TTE during the $\dot{V}O_{2peak}$ test did not change significantly between MC phases. This result is consistent with previous studies that used incremental (20, 26) or fixed-intensity tests (19, 21). In contrast, one study found reduced PO and a slower time during an 8-km time trial in the MLP compared to the mid-follicular phase, despite no changes in physiological variables (i.e., HR, $\dot{V}O_2$) (51). Whether these conflicting findings are related to the higher sensitivity of time trials than TTE-tests (52) or other factors is unknown. Concurrently, our data showed no effect of MC phase on HR_{peak}, BLa_{peak} or RPE_{peak}. Furthermore, the secondary analysis resulted in no significant associations between TTE, HR_{peak}, BLa_{peak} or RPE_{peak} and serum sex hormone concentrations or E:P ratio. Overall, it seems unlikely that TTE or any of the associated physiological variables at maximal effort are affected by MC phase or hormonal fluctuations between MC phases.

Another variable contributing to endurance performance is the ability to sustain a high $\%\dot{V}O_{2max}$ at the lactate threshold (1). Our results indicated that neither running velocity, $\%\dot{V}O_{2peak}$ nor HR at OBLA changed between MC phases or were associated with sex hormone concentrations. Interestingly, ~25% of the recorded tests in the current sample did not reach the 4 $mmol \cdot L^{-1}$ cutoff for the assessment of OBLA before reaching an RPE >17. While it is widely accepted that the blood lactate concentration at maximal lactate steady state can vary widely among individuals (39), there is little research investigating the suitability of existing threshold criteria in trained women specifically. From the limited literature available, the 4 $mmol \cdot L^{-1}$ blood lactate threshold has been shown to have high reliability/reproducibility (r=0.93) in trained women and men (53), and was therefore applied for this investigation. In comparison, Mattu et al. (21) utilized a multi-day protocol for the assessment of maximal lactate steady state and similarly reported no effect of MC phase. $\%\dot{V}O_{2max}$ at the lactate threshold is influenced by the rate of glycolysis in the active muscles (1, 54). Although studies looking at the isolated effects of E2 and P4 have demonstrated noticeable effects on substate

utilization, a recent meta-analysis revealed that substrate utilization was not affected by MC phase at rest or during moderate intensity exercise (13). When these findings are considered alongside the results of the present study, there appears to be limited evidence that hormonal fluctuations across the eumenorrheic MC are potent enough to provoke measurable changes to submaximal exercise performance metrics in endurance-trained women.

No significant effect of MC phase on RE was found, both when expressed as an absolute value or relative to body mass. Previous literature investigating the influence of MC phase on RE is limited and conflicting, as both an improved and reduced RE have been found in MLP when compared to EFP (26, 28). Notably, these studies relied on calendar-based counting to establish MC phases and did not clearly describe the inclusion/exclusion criteria of their participant group. In theory, a reduced RE in MLP could be supported by a shift in the thermoregulatory set point associated with elevated P4 during the luteal phase and the corresponding circulatory and metabolic strain (26, 55). However, performance differences corresponding to the increased core body temperature in MLP have only been shown in hot and/or humid environments, and it is generally agreed there is no significant influence on performance in temperate conditions (55, 56). Other physiological variables that may affect RE include $\dot{V}O_{2max/peak}$, [BLa-], and body mass (57), all of which remained stable across the MC in the current study. External factors known to affect RE, including pre-exercise diet, footwear and running surface, were all controlled for. Concurrently, our analysis did not reveal any association between the serum concentrations of E2, P4, T or E:P ratio in the different MC phases and RE. Thus, it appears that MC phase does not significantly influence RE.

In the current study, a 30-s all-out double-poling test was used to assess the maximal anaerobic power. MPO_{30s} and PPO_{30s} remained stable across MC phases in this study, which is consistent with findings summarized in a recent review (58). Interestingly, our secondary

388 analysis revealed a positive association between absolute serum T concentration and PPO_{30s}
389 and MPO_{30s}, irrespective of MC phase. The performance-enhancing effects of T (i.e., improved
390 strength and power) are well documented in men (59), yet there is surprisingly little
391 information on the effect of T on physical performance in trained women (60). Two previous
392 studies have demonstrated positive relationships between serum T levels and explosive power
393 (61) and sprint- and middle-distance running performance (62). Conversely, a recent review
394 was unable to support an association between T and muscular strength and performance in
395 women, possibly due to a lack of high-quality studies (60). While the aforementioned studies
396 have undertaken analysis across individuals, studies investigating within-individual changes in
397 T across the MC are limited and inconclusive (29, 63). Speculatively, T could influence MPO_{30s}
398 /PPO_{30s} via several neuromuscular or behavioral pathways (i.e. increased motivation or
399 competitiveness) (29, 59). However, further research is required to corroborate these
400 mechanisms in eumenorrheic women.
401
402 To our knowledge, this is the first study to include repeated $\dot{V}O_{2peak}$ measurements over
403 two cycles of testing. On an individual level, the EFP–MLP changes in $\dot{V}O_{2peak}$ were largely
404 inconsistent between individuals and across cycles. Previous research has also observed intra-
405 individual variability across cycles, with less than 30% of individuals showing directionally
406 consistent changes in endothelial function across two MCs (42). Although we did not detect an
407 effect of MC phase on $\dot{V}O_{2peak}$ at a group level, several participants responded consistently over
408 two cycles demonstrating that various individual patterns of response could possibly exist (i.e.
409 EFP-positive, MLP-positive, no effect, etc.). This is a notion that has also been described by
410 Veen Reen and Kieser in a larger cohort (64). Nonetheless these individual observations should
411 be interpreted with caution, as they reflect a limited number of individuals over just two cycles
412 and the observed variability could be attributed to numerous random and non-MC related

413 factors (65). Accordingly, whether these individual patterns would persist if a third or fourth
414 cycle were included is unknown. In addition, classification focused on the changes from EFP
415 to MLP and was heavily dependent on an estimated threshold, for which there are many
416 methods to consider (43, 66). Future studies are encouraged to measure responses over more
417 than two cycles with larger sample sizes to identify more conclusively if there are indeed
418 reproducible MC-phase traits both within and between individuals.
419
420 **Methodological Considerations**
421 The current study used the rigorous gold-standard methodology as described by
422 Schaumberg et al. (31) and Smith et al. (67) for the determination and verification of three
423 distinct MC phases. The selected phases (EFP, OP, MLP) represent distinct hormonal
424 environments which were hypothesised to influence the performance-determining variables.
425 However, this "three-phase model" does not account for the dynamic hormonal changes
426 occurring between phases (68). For instance, the late-luteal phase, a window of rapid hormonal
427 decline when cycle-related symptoms are often prevalent, was not included in this study. Thus,
428 we cannot be sure if MC related changes in the outcome variables would have been observed
429 if alternative timepoints were investigated or if notable day-to-day changes occurring between
430 these predefined phases.
431
432 It is reasonable to assume that some selection bias may have occurred in this study.
433 That is, females with severe MC symptoms may be less inclined to volunteer for a study in
434 which they are required to perform vigorous exercise on specific days of the MC. Alternatively,
435 they may choose to use hormonal contraception to regulate their symptoms. Recent research
436 published by our group provides support for the latter possibility, with athletes reporting that
437 the most common reason for hormonal contraceptive use was MC manipulation and the

attenuation of the accompanying negative symptoms (34). MC pain has also been related to avoidance of physical activity (69) and perceived reductions in performance (63). Interestingly, Dam et al. (63) reported that MC-related changes in psychological and physical wellbeing (i.e., perceived pain) were better predictors for variations in power performance than hormonal fluctuations. This may indicate that the participants in the current study represent a group with milder symptoms than the broader population, possibly influencing their sensitivity to cycle-related changes in performance-determining variables. Verifying this assumption would be methodologically and ethically challenging, but future research should consider how participation selection bias might influence female athlete research outcomes.

Finally, the external validity of this study should be considered. In an effort to tightly control for MC phase, this study included only eumenorrheic athletes, which may represent as little as 20% of the female athlete population (i.e. ≈60% of female athletes use hormonal contraceptives and up to 50% of non-hormonal contraceptive users may experience menstrual dysfunctions) (32, 34, 70). Additionally, test protocols were lab-based, which do not encompass the complexities of "real-world" performance. While we believe this level of control is necessary to answer some of the fundamental questions related to MC phase and endogenous hormones, future research could consider study designs that are inclusive to a broader scope of the population (i.e., hormonal contraceptive users and non-eumenorrheic athletes).

CONCLUSIONS

The present study found no influence of MC phase on the main determinants of endurance performance, such as $\dot{V}O_{2peak}$, $\%\dot{V}O_{2peak}$ at OBLA, RE and MPO_{30s}, in eumenorrheic endurance-trained women. Moreover, no significant associations were observed between the absolute concentrations of E2, P4 or E:P ratio measured in the various MC phases. However,

T was positively associated with MPO_{30s} and PPO_{30s}. The EFP–MLP changes in $\dot{V}O_{2peak}$ were inconsistent between-individuals and across cycles and no phase-specific patterns for improved or reduced performance-determining variables were observed on a group level. Given these findings, researchers should avoid excluding female participants from studies investigating responses to similar performance-determining variables based on the idea that MC phase will influence the outcomes on a group level.

Acknowledgements

The authors thank the participants for their participation, enthusiasm, and commitment to this study. The authors also thank Klavs Madsen for his collaboration and support at the Norwegian School of Sport Science, the University Hospital of Northern Norway for their support with processing and analyzing blood samples (Guri Grimnes, Ole Martin Fuskevåg, Silja Breivik) and the bioengineers and laboratory technicians for their assistance during the study period (Hege Nymo Østgaard, Madeleine-Emilie Killingstad).

This study was funded by the Tromsø Research Foundation (Project-ID: 19_FENDURA_BW) and UiT The Arctic University of Norway. The submission and page charges for this article have been funded by a grant from the publication fund of UiT The Arctic University of Norway.

Conflicts of interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The results of the study are presented clearly, honestly, and without fabrication, falsification, or in- appropriate data manipulation. The results of the present study do not constitute endorsement by the American College of Sports Medicine.

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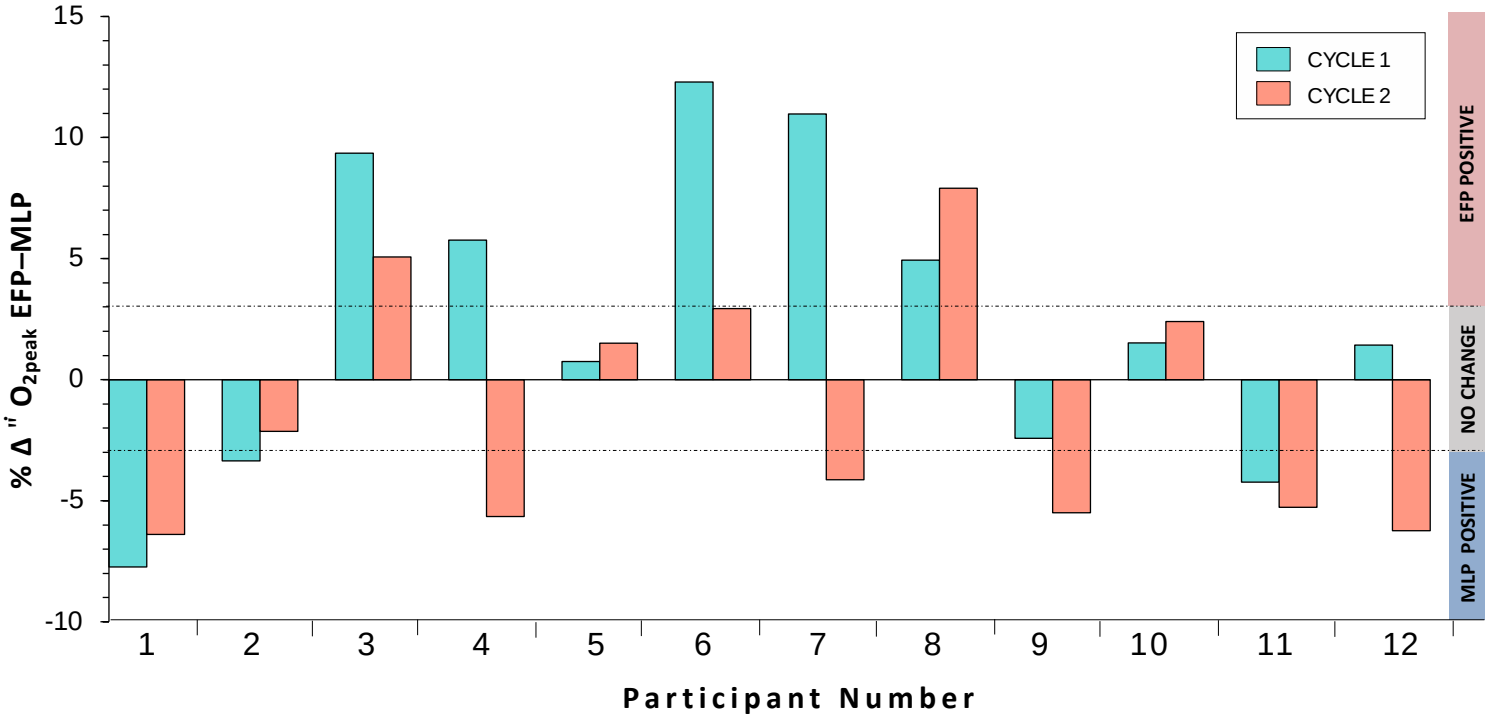
Supplementary Digital Content:

Supplementary Figure 1. (File Type .PDF)

Supplementary Figure 1: Consistency of $\dot{V}O_{2peak}$ outcomes between early follicular phase and mid luteal phase over two menstrual cycles.



Supplementary Figure 1: Threshold-based classification of percent change in peak oxygen uptake ($\dot{V}O_{2peak}$, $mL \cdot kg^{-1} \cdot min^{-1}$) between early follicular phase (EFP) and mid luteal phase (MLP) for 12 participants over two repeated cycles of testing. Dotted horizontal lines represent a standard measurement error threshold (±3%). EFP positive, higher $\dot{V}O_{2peak}$ in EFP compared to MLP. MLP positive, higher $\dot{V}O_{2peak}$ in MLP compared to EFP. No change, the change in $\dot{V}O_{2peak}$ from EFP to MLP is within the standard threshold of possible measurement error.