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# Menstrual cycle phase does not influence muscle protein synthesis or whole-body myofibrillar proteolysis in response to resistance exercise

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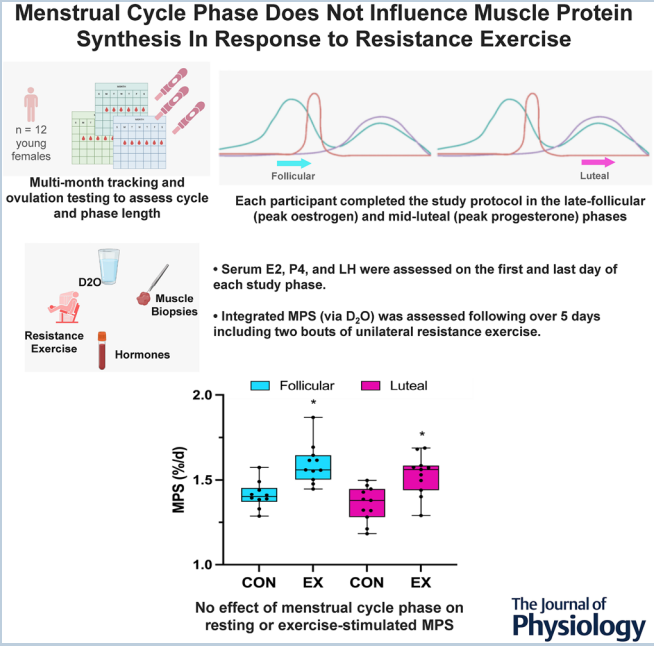


**Abstract figure legend** In this study, we used best-practice methodology to establish menstrual cycle phases of women. We used stable isotope methodologies to assess muscle protein synthesis (MPS) in the mid-follicular and mid-luteal phases of their menstrual cycles. One leg performed two bouts of resistance exercise with the contralateral rested leg acting as a control; this was reversed in the opposite menstrual cycle phase. We also assessed whole-body protein myofibrillar protein breakdown (MPB). We saw, as expected, that resistance exercise stimulated MPS but that there was no effect of menstrual cycle phase on the MPS or MPB responses. There appears to be no anabolic 'advantage' to performing resistance exercise in any particular phase of a woman's menstrual cycle.



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**Abstract** It has been hypothesised that skeletal muscle protein turnover is affected by menstrual cycle phase with a more anabolic environment during the follicular *vs*. the luteal phase. We assessed the influence of menstrual cycle phase on muscle protein synthesis and myofibrillar protein breakdown in response to 6 days of controlled resistance exercise in young females during peak oestrogen and peak progesterone, using stable isotopes, unbiased metabolomics and muscle biopsies. We used comprehensive menstrual cycle phase-detection methods, including cycle tracking, blood samples and urinary test kits, to classify menstrual phases. Participants ($n = 12$) completed two 6 day study phases in a randomised order: late follicular phase and mid-luteal phase. Participants performed unilateral resistance exercise in each menstrual cycle phase, exercising the contralateral leg in each phase in a counterbalanced manner. Follicular phase myofibrillar protein synthesis (MPS) rates were $1.33 \pm 0.27\%\ h^{-1}$ in the control leg and $1.52 \pm 0.27\%\ h^{-1}$ in the exercise leg. Luteal phase MPS was $1.28 \pm 0.27\%\ h^{-1}$ in the control leg and $1.46 \pm 0.25\%\ h^{-1}$ in the exercise leg. We observed a significant effect of exercise ($P < 0.001$) but no effect of cycle phase or interaction. There was no significant effect of menstrual cycle phase on whole-body myofibrillar protein breakdown ($P = 0.24$). Using unbiased metabolomics, we observed no notable phase-specific changes in circulating blood metabolites associated with any particular menstrual cycle phase. Fluctuations in endogenous ovarian hormones influenced neither MPS, nor MPB in response to resistance exercise. Skeletal muscle is not more anabolically responsive to resistance exercise in a particular menstrual cycle phase.

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## Key points

- It has been hypothesised that the follicular (peak oestrogen) *vs*. the luteal (peak progesterone) phase of the menstrual cycle is more advantageous for skeletal muscle anabolism in response to resistance exercise.
- Using best practice methods to assess menstrual cycle status, we measured integrated (over 6 days) muscle protein synthesis (MPS) and myofibrillar protein breakdown (MPB) following resistance exercise in females ($n = 12$) in their follicular and luteal phases.
- We observed the expected differences in oestrogen and progesterone concentrations that confirmed our participants' menstrual cycle phase; however, there were no notable metabolic pathway differences, as measured using metabolomics, between cycle phases.
- We observed that resistance exercise stimulated MPS, but there was no effect of menstrual cycle phase on either resting or exercise-stimulated MPS or MPB.
- Our data show no greater anabolic effect of resistance exercise in the follicular *vs*. the luteal phase of the menstrual cycle.

**Lauren Colenso-Semple** received her PhD from McMaster University, working with Professor Stuart Phillips. Lauren has an MS from the University of South Florida and a BA from SUNY at Buffalo. Lauren's work focuses on the impact of exercise, nutrition and hormones on skeletal muscle with a specific focus on female endocrinology. Lauren is a certified personal trainer and group fitness instructor and has worked with hundreds of clients in person and online, including recreational lifters, mums-to-be, aspiring powerlifters and physique athletes.

## Introduction

Premenopausal females are frequently excluded from exercise physiology research, with an often-cited reason being the potential for menstrual cycle ovarian hormones to influence metabolic, performance or muscle-based outcomes (Costello et al., 2014). The assumption that the menstrual cycle or hormonal contraceptive use influences outcomes has led, in part, to a widespread sex-based bias in the exercise physiology literature (Lew et al., 2022). Although the primary purpose of ovarian hormones [oestradiol, E2, and progesterone, P4) is for reproductive function, it has been proposed that oestrogen signalling may be involved in pathways and processes that influence muscular adaptations to exercise (Ikeda et al., 2019; Sitnick et al., 2006; Toth et al., 2001). One study reported that ovarian hormones inhibit protein turnover and muscle growth in ovariectomised rats (Toth et al., 2001). Additionally, ovariectomy (OVX) has been shown to impair the regrowth of atrophied skeletal muscle, and oestrogen regulates repair and remodelling of muscle (Sitnick et al., 2006). However, the OVX model does not translate to humans (at least those not undergoing a hysterectomy), and ovarian hormonal influences on skeletal muscle across the menstrual cycle in humans are poorly understood. Nonetheless, these data (Ikeda et al., 2019; Sitnick et al., 2006; Toth et al., 2001) are often cited as the foundation for a hypothesis that fluctuations in ovarian hormones influence skeletal muscle adaptations to exercise in humans (Kissow et al., 2022; Oosthuyse et al., 2023).

It has been speculated that alterations in menstrual cycle hormones influence protein catabolism, which is a process that has been proposed to be enhanced in the luteal compared to the follicular phase of the menstrual cycle (Oosthuyse et al., 2023), ostensibly as a result of progesterone antagonising the anabolic effect of oestrogen (Van Every et al., 2024); however, whether this assertion, based primarily on whole body stable isotope-measured amino acid oxidation in humans, is muscle-specific remains unknown. It has also been speculated that muscle regeneration is greater during the follicular phase when oestrogen is higher and progesterone is lower (Oosthuyse et al., 2023). Despite such speculation, surprisingly few human trials have investigated the effects of menstrual cycle phase on muscle anabolism. Miller and colleagues reported similar postexercise muscle protein synthesis rates between two groups of females in the follicular or luteal phases of naturally cycling females despite substantial differences in serum E2 and P4 levels (Miller, 2006). However, in that study, the small sample sizes, the acute nature of the measurements of protein synthesis and the between-group study design limit the broader interpretation of the work (Miller, 2006) and, in our view, further investigation is warranted. Variations in oestrogen receptor RNA and protein content in muscle have also been reported to occur across the menstrual cycle; however, the significance of these data are unknown because, to date, scant data show an associated phenotypic outcome (Ekenros et al., 2017).

The present study aimed to investigate muscle protein synthesis and myofibrillar proteolysis in response to resistance exercise in naturally menstruating females. Subjects were assessed during their late follicular phase (i.e. highest E2 concentration) and their mid-luteal phase (i.e. highest P4 concentration). We employed a cross-over design, unilateral resistance exercise (switching legs between phases in a counterbalanced manner) and integrated 5 day assessments of protein turnover, as well as conducting unbiased metabolomics to look for cycle-specific metabolite patterns. Despite limited previous work, we aimed to test the hypothesis that muscle protein synthesis would increase in response to resistance exercise in both phases but to a greater extent in the follicular phase as a result of higher E2 compared to the luteal phase (i.e. higher P4), as speculated (Oosthuyse et al., 2023).

## Methods

The study was approved by the Hamilton Integrated Research Ethics Board (project number: 14 067) and conformed to the standards for the use of human subjects in research as outlined by the Canadian Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans – TCPS 2, 2022 (https://ethics.gc.ca/eng/policy-politique_tcps2-eptc2_2022.html) and the Declaration of Helsinki (https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects/). Each participant was informed of the purpose of the study, experimental procedures and potential risks before written informed consent was obtained. The trial was registered with the National Institutes of Health at http://www.clinicaltrials.gov repository as NCT05347667.

### Participants

Healthy young females ($n = 12$) were recruited for the study. Eligible participants were between the ages of 18 and 30 years, non-smokers or users of tobacco products, and in good health (as determined by a medical screening questionnaire). All participants reported having a regular menstrual bleed, as determined by a tracking app, and had not used any form of hormonal contraceptive for at least 6 months prior to the study. Participants were excluded if they: (1) suffered from an orthopaedic, cardiovascular, pulmonary, renal, liver, infectious disease, immune, metabolic or gastrointestinal disorder

**Table 1. Participant characteristics**

| Characteristic | Value |
|---|---|
| Age (years) | 19 ± 1 |
| Height (cm) | 165 ± 1 |
| Body mass (kg) | 59.3 ± 5.1 |
| Body mass index (kg/m$^2$) | 21.8 ± 1.9 |
| Menstrual cycle length (days) | 31 ± 3 |
| Menstrual cycle range (days) | 27–35 |
| Ovulation (day) | 17 ± 3 |
| Length of follicular phase (days) | 17 ± 3 |
| Length of luteal phase (days) | 15 ± 3 |
| Ratio of follicular to luteal phase length (days) | 0.92 ± 0.26 |
| Lean mass (kg)* | 38.9 ± 3.0 |
| Muscle mass (kg)** | 18.3 ± 1.3 |

Values are the mean ± SD.
*Derived from DXA.
**Derived from $D_3$-creatine.

expected to impact study outcomes; (2) took medications known to affect protein metabolism (i.e. corticosteroids, non-steroidal anti-inflammatory drugs or high strength acne medication, or testosterone replacement); (3) used tobacco or tobacco-related products (smoking or vaping); and (4) had been diagnosed with a menstrual cycle disorder, polycystic ovarian syndrome or endometriosis. Participant characteristics are shown in Table 1.

A sample size of 12 subjects was determined based on an *a priori* power analysis calculated in G*power, version 3.1.9.6 (Franz Faul, Kiel University, Germany) based on previous trials of a similar nature (target alpha of 0.05 and power of 0.80) with a small effect size of 0.2 for change of ∼20% in muscle protein synthesis. Not knowing the magnitude of the difference to expect, with no prior work in humans, we based the sample on a change that would be physiologically relevant using our previous data as the basis for the calculation.

## Study overview

Participants completed two 6 day study phases in a randomised order: late follicular phase (5 days prior to predicted ovulation, based on the timing of positive urinary ovulation tests from at least two cycles) and mid-luteal Phase (5 days following a positive urinary ovulation test). The study protocols were completed in different menstrual cycles. A schematic of the study protocol is shown in Fig. 1.

Participants completed a general health questionnaire to indicate their current health status and medication use to ensure eligibility for the study. Height and body mass were assessed using a calibrated stadiometer and scale. Participants underwent a dual X-ray absorptiometry (DXA) (GE-Lunar iDXA; Aymes Medical, Toronto, ON, Canada) scan to assess body composition. DXA-derive lean mass was used to determine $D_2O$ dosing. Unilateral knee extension 10 repetition maximum was assessed for each leg to determine the starting load for subsequent study visits.

Participants arrived for the first study visit after an overnight fast. Following a pregnancy test, they underwent a full-body DXA scan to assess body composition. They provided a baseline saliva sample (to obtain baseline body water enrichment; see below), a baseline urine sample (to measure $D_3$-creatinine enrichment; see below), a blood sample (to assess serum hormones) and a baseline muscle biopsy from the vastus lateralis of the control leg. The control leg was randomly determined for phase one and the contralateral leg served as the control for phase two. Participants were given three (1.25 mL kg$^{-1}$ lean mass) aliquots of 70 atom % $D_2O$ to consume 30 min apart. An oral dose of 30 mg $D_3$-Cr was included in the third aliquot of $D_2O$ to assess skeletal muscle mass as previously described in detail (Cegielski et al., 2021, 2022). All loading doses were consumed in the laboratory. Finally, participants performed three sets of 10 unilateral knee extensions to volitional fatigue, defined as an inability to complete a repetition through the full range of motion. If the participant completed more than 12 or less than eight repetitions, the weight was adjusted, up or down, accordingly. Prior to leaving the laboratory, participants were provided with four additional aliquots of $D_2O$ and four salivettes for saliva collection. They were instructed to collect a saliva sample (prior to consuming food or water) and to consume one dose of $D_2O$ every morning for the next 4 days. They completed written logs to indicate the time each saliva sample was collected and each $D_2O$ dose was consumed. Participants returned to the laboratory 48 h after visit 1 to provide a urine sample and perform three additional sets of unilateral knee extensions as outlined above. Participants returned to the laboratory 72 h after visit 1 to provide a urine sample and 18 h prior to the scheduled visit 5 to consume 10 mg of $D_3$-3MH dissolved in water. After an overnight fast, participants reported to the laboratory for the final visit. Muscle biopsies were taken from the exercise and control legs. Blood samples were collected hourly for 5 h to assess plasma $D_3$-3MH and measure whole-body myofibrillar proteolysis as described previously (Cegielski et al., 2021, 2022).

## Blood analysis

Blood samples were taken from an antecubital vein and collected in a serum separating tube and EDTA tubes to isolate serum and plasma, respectively. All blood tubes were centrifuged at 1500 *g* for 10 min at 4°C prior to serum and plasma being separated into cryotubes and

frozen at –80°C until further analysis. Blood samples were analysed using the Ortho Vitros MicroWell (QuidelOrtho Corp., San Diego, CA, USA), employing the VITROS 5600 Integrated System (QuidelOrtho Corp.) that provides enhanced chemiluminescence detection for serum E2 (pmol $L^{-1}$; by competitive immunoassay; inter-assay CV <4%), P4 (nmol $L^{-1}$; by competitive immunoassay; inter-assay CV <6%) and luteinising hormone (LH) (IU $L^{-1}$; by non-competitive immunometric assay; inter-assay CV <5%) by the Hamilton Regional Laboratory Medicine Program, as well as $D_3$-3-methyl-histidine enrichment as described previously (Cegielski et al., 2021, 2022).

## Deuterium oxide

The incorporation of deuterium (as $D_2O$) into muscle protein-bound alanine was assessed to quantify muscle protein synthesis rates (Wilkinson et al., 2014). The protocol consisted of one loading day and four maintenance days with the goal of enriching and maintaining the body water pool. Participants ingested three doses (1.25 mL $kg^{-1}$ lean body mass) of 70% $D_2O$ (Cambridge Isotope Laboratories, Andover, MA, USA) every 30 min on the loading day and one dose per maintenance day.

## Muscle biopsies

Muscle biopsy samples were obtained on seven occasions using a 5 mm Bergstrom needle modified for manual suction under 1% xylocaine local anaesthesia. The first biopsy site was ∼15 cm above the patella, and subsequent biopsy sites were spaced ∼3–5 cm apart. Biopsies were taken from the control limb pre-exercise (phase 1 visit 1) and the control and exercise limbs (phase 1 visit 5, phase 2 visit 1 and phase 2 visit 5). Visible connective and adipose tissue were dissected from each specimen prior to being snap-frozen in liquid nitrogen and stored at −80°C.

## Saliva analysis

Saliva samples were obtained by gently chewing on a cotton swab for 2–3 min until completely saturated with saliva. Salivettes were centrifuged at 1500 *g* for 10 min and diluted in doubly distilled water. Saliva samples were analysed for $^2H$ (D) enrichment by cavity ringdown spectroscopy (L2130-i; Picarro Inc., Santa Clara, CA, USA). Briefly, the $^2H$ (D) enriched saliva samples were diluted (1:50) with doubly distilled water and analysed using express mode (i.e. injected ten times; six wet flushes and four sample injections) with the average of the last three measurements used for analysis.

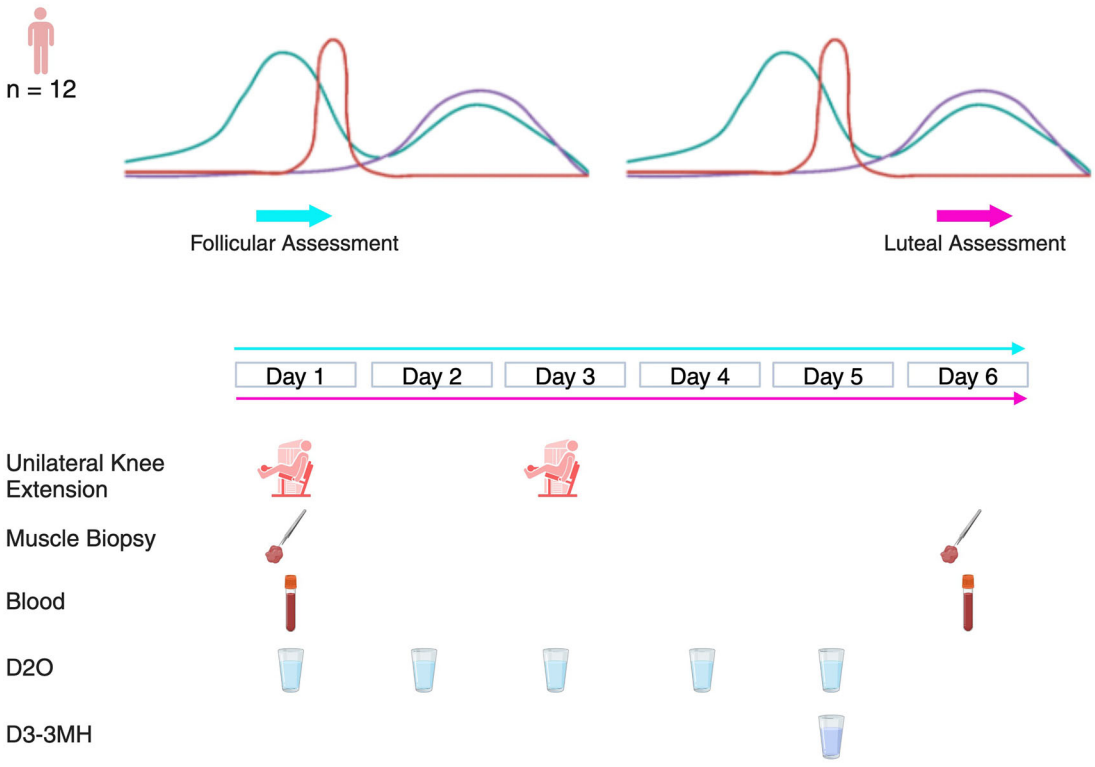


**Figure 1. Schematic depiction of the protocol that was repeated in each phase**
The exercised limb was randomly selected and was switched in a counterbalanced manner, as was the phase in which each participant began the protocol.

Measurements were corrected for machine drift and background enrichment, and the $^2$H (D) isotopic enrichments for saliva were converted to atom percent excess using standard equations:

$$\text{Atom percent excess (APE)} = \left[\frac{100 \times AR \times (\delta D \times 0.001 + 1)}{1 + AR\,(\delta D \times 0.001 + 1)}\right]$$

where AR represents the absolute ratio constant for deuterium based on the VSMOW standard and equates to 0.00015595.

## Myofibrillar extraction

Snap-frozen muscle samples were homogenised using 5 mm stainless steel beads in a 2 mL Eppendorf (2 × 40 s at 20 Hz; TissueLyser, Hilden, Germany) with 500 µL of fresh, ice-cold homogenisation buffer (25 mM Tris buffer [Tris-HCl, Trizma Base, doubly distilled $H_2O$ ($ddH_2O$) pH 7.2], 1 PhosStop Tablet (Roche, Basel, Switzerland), 1 complete (Roche) mini protease inhibitor tab, 100 µL of TritonX-100). Samples were centrifuged at 280 *g* for 10 min at 4°C to separate the sarcoplasmic and myofibrillar fractions. The myofibrillar fraction was purified by adding 500 µL of $ddH_20$, vortexing for 5 s and centrifuging at 280 *g* for 10 min at 4°C. Next, 1 mL of 0.3 M NaOH was added to the sample and vortexed before being placed in a heating block at 50°C for 30 min (vortex 5 s every 10 min) to solubilise the myofibrillar proteins. Samples were centrifuged at 12,300 *g* for 10 min at 4°C to pellet the collagen proteins, and the supernatant (containing the myofibrillar fraction) was placed in a 4 mL glass screw-top tube. Proteins were precipitated with 1 mL of 1 M perchloric acid and centrifuged at 770 *g* for 10 min at 4°C. After removing the supernatant, the myofibrillar protein pellet was washed twice in 70% ethanol (centrifuging at 280 *g* for 10 min at 4°C). Amino acids were liberated by adding 1 mL of Dowex resin (50WX8-100-200 mesh resin; Sigma-Aldrich, St Louis, MO, USA) and 1 mL of 1 M HCL before heating at 110°C for 72 h. The free amino acids were purified on cation-exchange columns, dried under vacuum in a rotary evaporator and reconstituted in 0.1 M HCl before gas chromatography-pyrolysis-isotope ratio mass spectrometry (MS) analysis.

## Integrated myofibrillar protein synthesis (MPS)

Ingestion of $D_2O$ was used to label newly synthesised myofibrillar proteins (Wilkinson et al., 2014). MPS rates were determined using the standard precursor-product method (McGlory et al., 2018; Stokes et al., 2020). Total body water (saliva) deuterium ($^2$H) enrichment (converted to its natural log) was used as a surrogate for plasma alanine labelling (precursor). The change in $^2$H enrichment (relative to $^1$H) of muscle alanine (product) over time was used to calculate the myofibrillar fractional synthesis rate (FSR).

## Metabolite classification

Plasma samples were analysed by Metabolon Inc. (Morrisville, NC, USA) using procedures described previously (Handelman et al., 2019; Shin et al., 2014) prepared using the automated MicroLab STAR® system (Hamilton Company, Reno, NV, USA). Recovery standards were added prior to the first step in the extraction process for QC purposes. Samples were extracted with methanol under vigorous shaking for 2 min (GenoGrinder 2000; Glen Mills Inc., Clifton, NJ, USA) to precipitate protein and dissociate small molecules bound to protein or trapped in the precipitated protein matrix, followed by centrifugation to recover chemically diverse metabolites. The resulting extract was divided into five fractions: two for analysis by two separate reverse phase/ultra performance liquid chromatography (UPLC)-MS/MS methods using positive ion mode electrospray ionisation (ESI), one for analysis by reverse phase/UPLC-MS/MS using negative ion mode ESI, one for analysis by hydrophilic interaction liquid chromatography (HILIC)/UPLC-MS/MS using negative ion mode ESI and one reserved for backup. Samples were placed briefly on a TurboVap® (Zymark; Sigma-Aldrich) to remove the organic solvent.

Metabolites were identified by automated comparison of the ion features in the experimental samples to a reference library of chemical standard entries that included retention time, molecular weight (*m*/*z*), preferred adducts, and in-source fragments, as well as associated MS spectra; these were curated by visual inspection for quality control using software developed at Metabolon (Metabolon Inc., Research Triangle Park, NC, USA). Total ion count data across the sampling interval of each metabolite (corresponding to the area under the peak in HPLC alone) were used as a surrogate for metabolite abundance. Metabolon quality practices are described extensively elsewhere (Handelman et al., 2019; Shin et al., 2014). Several types of controls were analysed in concert with the experimental samples: a pooled matrix sample generated by taking a small volume of each experimental sample served as a technical replicate; extracted water samples served as process blanks; and a cocktail of QC standards chosen not to interfere with the measurement of endogenous compounds were spiked into every analysed sample to monitor instrument performance and aid with chromatographic alignment. Instrument variability was determined by calculating the median relative SD for the standards that were added to each sample prior to injection into the mass spectrometers.

Overall process variability was determined by calculating the median relative SD for all endogenous metabolites (i.e. non-instrument standards) present in 100% of the pooled matrix samples. Experimental samples were randomised across the platform run, with QC samples spaced evenly among the injections.

### Creatinine enrichment

Samples were thawed at room temperature and had 250 µL of ice-cold acetonitrile added, then were vortexed, mixed and cooled on ice for 30 min. Samples were then centrifuged at 17,000 *g* for 20 min. The supernatant was filtered through a 0.2 µm filter and transferred to vials ready for HPLC-MS analysis using the same instrumentation and column as above. The flow was set to 0.2 mL $min^{-1}$, 60:40 (buffer A:B) isocratic flow, where buffer A was 100% acetonitrile and buffer B was ammonium acetate, pH 5.8. A standard curve using $D_3$-creatine was prepared for the determination of creatine concentration and enrichment [monitoring mass + hydrogen cation (M + H): 135.1], with a $D_3$-creatinine enrichment curve of 0–0.1% for the determination of $D_3$-creatinine enrichment (monitoring M + H: 114.1 and 117.1). The estimated creatine pool size was divided by 4.3 g $kg^{-1}$, which reflects the average concentration of creatine found in whole wet muscle tissue (Clark et al., 2014).

### Plasma $D_3$–3–methyl–histidine

Plasma samples were defrosted and centrifuged at 12,300 *g* for 3 min. A 0–10% $D_3$-3-methyl-histidine enrichment curve was prepared as a serial dilution. For this, 100 µL of plasma was de-proteinised using 1 mL of MeCN:MeOH (1:1). Samples were vortex mixed and incubated at −20°C for 1 h. Samples were centrifuged at 20,800 *g* for 5 min at 4°C. The supernatant was dried down in a Dri-Block heater (Bio-Techne Corp., Minneapolis, MN, USA) at <40°C using nitrogen gas. Samples were re-suspended using 100 µL of MeCN: ddH2O (65:35) and ready to be analysed using HPLC (Dionex Ultimate3000; Thermo Fisher Scientific, Waltham, MA, USA) MS (Q-Exactive; Thermo Fisher Scientific) with a Sequant ZIC-HILIC column (150 mm × 2.1 mm, 5 µm; Merck Millipore, Burlington, MA, USA). The flow was set to 0.4 mL $min^{-1}$ with an initial buffer gradient of 95:5 (buffer A:B), where buffer A was 10 mM ammonium formate (90:10 acetonitrile:ddH2O) with 0.1% formic acid and buffer B was 10 mM ammonium formate (50:50 acetonitrile:ddH2O) with 0.1% formic acid. After a 2.5 min hold at 95:5 (A:B), the buffer gradient was ramped to 100% buffer B over 15 min and held for 2.5 min before returning to 95:5 (A:B) and re-equilibrated for 10 min. Accurate mass single ion monitoring was performed for M + H: 170.09 (3MH) and 173.11 ($D_3$-3MH) to determine the enrichment of $D_3$-3MH. The enrichment ratios were log-transformed to determine the decay rates (*k*), representative of the rate of whole-body MPB (Sheffield-Moore et al., 2014).

### Statistical analysis

Data were analysed in SAS, version 9.4 (SAS Institute Inc., Cary, NC, USA) with a linear mixed model with phase (follicular or luteal) and leg (exercise or control) as within-subject factors using restricted maximum likelihood estimation with the Satterthwaite approach to estimating degrees of freedom. Observations were treated as nested within the subject. $P < 0.05$ was considered statistically significant. Data are presented as the means ± SD unless otherwise indicated.

## Results

### Participants

All participants had menstrual cycle phase lengths, ovulation and hormone concentrations that, according to our predefined criteria, allowed for them to be included in the study.

### Hormones

Serum E2, P4 and LH were assessed in both phases. Data are presented in Table 2 (and shown graphically in the Supporting information, Data Fig. 3). Changes in the concentrations of hormones followed expected patterns in accordance with each participant's menstrual cycle phase. There was, however, substantial variation in hormone concentrations.

### Metabolite profiling

Global metabolomic profiling of blood was used to explore changes in plasma metabolites at different phases of the menstrual cycle. The analysis identified 1376 unique biomolecules (1112 named biomolecules of known structure and 264 unnamed compounds). There were changes in free amino acids, their post-translationally modified derivatives and dipeptides, but no clear patterns or associations were observed between these markers and different phases of the menstrual cycle (Fig. 2). We also include other known metabolites and analyses showing some trends in metabolites and cluster analysis of certain metabolites related to progesterone metabolism (see Supporting information, Fig. 1A and B) and boxplots of metabolites (10.6084/m9.figshare.27023518). Broadly, we did not observe any remarkable changes in metabolites

**Table 2. Serum hormone concentrations**

| Hormone | Follicular (–5 days) | Late follicular | Luteal (+5 days) | Luteal (+10 days) |
|---|---|---|---|---|
| Oestradiol (pM) | 251 ± 109 | 605 ± 301 | 583 ± 214 | 582 ± 218 |
| Progesterone (nM) | 7 ± 5 | 13 ± 12 | 34 ± 19 | 40 ± 19 |
| Luteinising hormone (IU $L^{-1}$) | 8 ± 4 | 33 ± 32 | 12 ± 11 | 6 ± 4 |

Values are the mean ± SD.

related to each menstrual phase, but some notable individualised trends (10.6084/m9.figshare.27023518; see also Supporting information, Fig. 1A and B). Figure 3 highlights a principal components analysis of the metabolites in Fig. 2, showing that individuals had patterns that were somewhat clustered, and menstrual cycle phase exhibited no consistent pattern in metabolites, showing no menstrual phase-specific trend that characterised any aspect of metabolism.

## MPS

The mean ± SD body water enrichment of $D_2O$ was 0.34 ± 0.03 APE in the follicular phase and 0.37 ± 0.05 APE in the luteal phase (see Supplemental Fig. 2). The mean myofibrillar FSR in the follicular phase was 1.33 ± 0.27% $h^{-1}$ in the control leg and 1.52 ± 0.27% $h^{-1}$ (0.27) in the exercise leg. The mean myofibrillar FSR in the luteal phase was 1.28 ± 0.27% $h^{-1}$ in the control leg and 1.46 ± 0.25% $h^{-1}$ in the exercise leg. There was a significant effect of exercise ($P < 0.001$), but no effect of phase ($P = 0.213$) and no interaction effect ($P = 0.299$). The results are presented in Fig. 4.

## Myofibrillar protein breakdown (MPB)

The mean ± SD rate ($k$) of whole-body MPB was 0.015 ± 0.005 in the follicular phase and 0.022 ± 0.02 in the luteal phase ($P = 0.244$). The results are presented in Fig. 5.

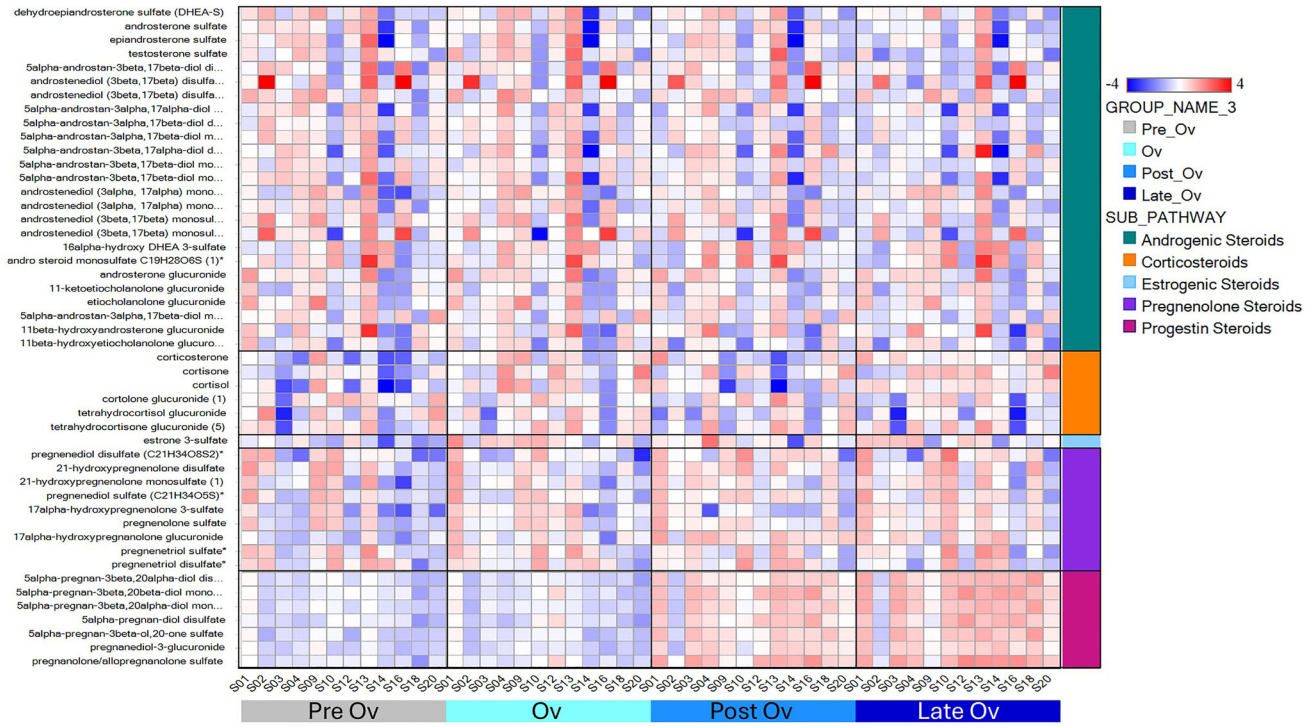


**Figure 2. Hierarchical clustering analysis (HCA) of steroid hormones and metabolites**
Columns represent individual participant data grouped by menstrual cycle phase: Pre_Ov, pre-ovulation (early follicular); Ov, ovulation (late follicular); Post_Ov, post-ovulation (early luteal); Late_Ov, late ovulation (late luteal). Rows are individual metabolites (with the sidebar colour-coded to highlight metabolite subpathway clusters).

## Discussion

Following best practice methodologies to establish regular menstrual cycles, ovulation and menstrual cycle phases, we did not observe differences in MPS or whole-body MPB between menstrual cycle phases. We also observed no remarkable trends in metabolites that would indicate a menstrual cycle phase-specific pattern. Our data do not support the thesis that endogenous ovarian hormone fluctuations influence the anabolic response of muscle to resistance exercise.

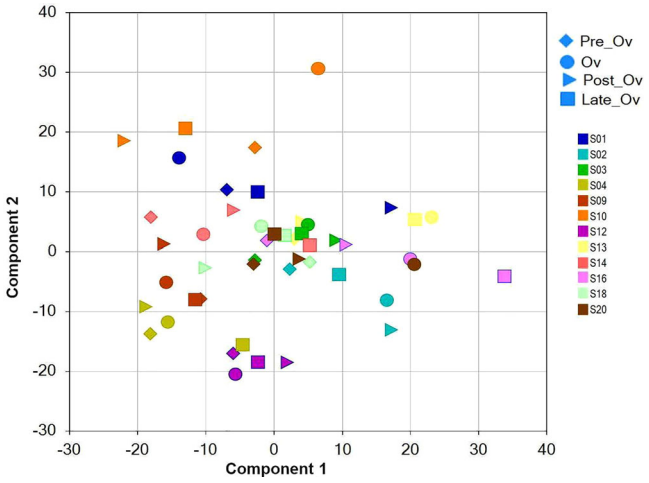


**Figure 3. Principal components analysis of data from Fig. 2**
Each coloured symbol represents an individual participant during an individual menstrual phase, identified by the shape. Pre_Ov, pre-ovulation (early follicular); Ov, ovulation (late follicular); Post_Ov, post-ovulation (early luteal); Late_Ov, late ovulation (late luteal).

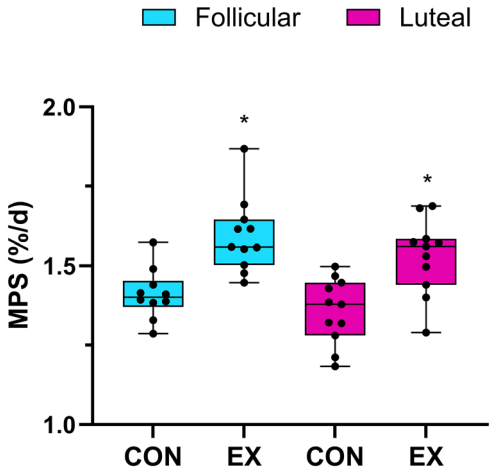


**Figure 4. Integrated muscle protein synthesis in follicular and luteal phases**
There was no significant effect of menstrual cycle phase or interaction between phases and conditions (EX *vs.* CON; all $P > 0.4$). *Significant difference (main effect) between EX and CON ($P < 0.001$).

Our findings broadly align but expand on the conclusions of an earlier study by Miller (2006), who found no influence of menstrual cycle phase on acute resting or postexercise muscle protein synthesis using a between-subject experimental design. Crucially, previous works assessing the influence of the menstrual cycle phase and oral contraceptives (Hansen et al., 2011; Miller, 2006) have used short-term (hours) infusions of isotopes to estimate muscle protein synthesis. As such, it is noteworthy that prior data were collected over a few hours post-exercise, with the link to eventual effects on muscle growth being tenuous (Mitchell et al., 2015). By contrast, recent developments in the use of deuterated water to assess integrated diurnal muscle protein synthesis showed improved associations with muscle hypertrophy (Damas et al., 2016). Our data provide an assessment of muscle protein synthesis over 5 days in free-living conditions with two controlled bouts of resistance exercise. Using this method of assessing MPS incorporates days of exposure to RE and the overall hormonal milieu (Damas et al., 2016) and would have incorporated peak oestrogen and peak progesterone concentrations. Albeit acknowledging that MPS is not a measure of net muscle protein accretion (or muscle hypertrophy), menstrual cycle phase did not affect muscle protein synthesis.

We also assessed whole-body myofibrillar proteolysis using labelled $D_3$–3–methyl–histidine as originally described (Sheffield-Moore et al., 2014) and modified using an adapted combined oral stable isotope assessment of muscle (COSIAM) method (Cegielski et al., 2021, 2022). We acknowledge that this measure is not able to isolate the effect of the exercise *per se*, but we noted no phase-specific differences in the decay rate ($k$), which was in agreement with the concentrations of 3-methylhistidine measured in the blood (data not shown). We also explored the metabolic milieu using metabolomics approaches

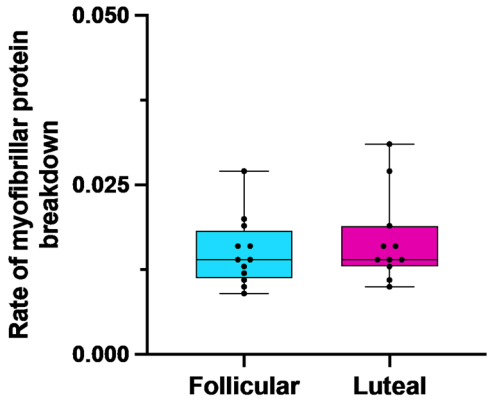


**Figure 5. Whole-body rates ($k$) of myofibrillar protein breakdown (MPB) in the follicular and luteal phases of the menstrual cycle**

(Figs 2 and 3; see also Supporting information, Data Fig. 1A and B) and saw no uniform pattern of changes in metabolites specific to each menstrual cycle phase. Our results are not overly different from previous work using metabolomics in characterising menstrual cycle phase (Draper et al., 2018; Wallace et al., 2010) in which few metabolites showed phase-specific patterns. We obtained data on more metabolites than reported in previous work (Draper et al., 2018; Wallace et al., 2010), used different methodologies and utilised resistance exercise as an intervention. However, Wallace et al. (2010) also performed a principal component analysis and, like ourselves, found no differences based on menstrual phase. Thus, we see no evidence to suggest any menstrual cycle phase-specific effect on metabolism, in line with our meta-analysis (D'Souza et al., 2023), muscle anabolic processes or myofibrillar proteolysis, as well as no support for the contention that the follicular phase is associated with a greater potential for muscle anabolism (Oosthuyse et al., 2023) or that the luteal phase is associated with greater catabolism. In summary, if the link between our measures of MPS and MPB to an eventual phenotype can be made, we see no rationale for planning RE training around a proposed advantage of menstrual-related hormones that is specific to one or another menstrual cycle phase.

Recently, there have been calls to measure ovarian hormones as opposed to assuming (guessing) their concentrations to improve the accuracy of conclusions on research in females (Burden et al., 2024). We used best practice methods (Elliott-Sale et al., 2021) to assess our participants' menstrual cycle phase by tracking to determine cycle length and ovulation detection to identify phase length. Findings are often attributed to an assumed hormonal profile (Burden et al., 2024) that may or may not be present. As highlighted in meta-analyses by McNulty et al. (2020) and Blagrove et al. (2020) and a systematic review by Meignié et al. (2021), this area of research is, regrettably, populated by low-quality studies because of substantial methodological issues (Elliott-Sale et al., 2021), including the absence of cycle length tracking, ovulation assessments and blood hormone measurements. Indeed, we noted substantial inter-individual variability in hormone levels in our participants (Table 2 and Supplemental Data Fig. 3) despite the standardised assessment time points, which highlights the strength of the within-subject model employed in this study. Our data also demonstrate the importance of assessing ovarian hormone levels at multiple time points across the menstrual cycle. Many sources cite an average menstrual cycle length of 28 days, with the onset of the luteal phase on day 14 (Reed & Carr, 2018); however, we observed substantial inter-individual variability in cycle and phase length. Menstrual cycle length ranged from 27 to 35 days, with only one participant out of 12 having a cycle length of 28 days. The timing of the LH surge indicative of ovulation ranged from day 13 to day 26. The length of the follicular phase ranged from 13 to 26 days and the length of the luteal phase ranged from 11 to 17 days. The follicular phase is often characterised by high E2 and the luteal phase is defined by high P4; however, it is important to acknowledge that, at the beginning of the follicular phase (the onset of menstruation), both hormones are low. In addition to a P4 peak in the luteal, there is a secondary E2 peak during the mid-luteal phase. Indeed, the mean E2 levels were similar and not statistically different (Table 2) during the late follicular phase and the mid-luteal phase.

Nonetheless, progesterone was, as expected, elevated in the luteal phase (Table 2), which has been speculated to antagonise the ostensible anabolic effect of oestrogen (Van Every et al., 2024). We also used a 5 day exercise and data collection period over which to measure anabolism and also captured peak oestrogen and peak progesterone (as well as elevated oestrogen) concentrations. Our approach provides the most ecologically valid test of the thesis that one particular phase of the menstrual cycle with its incumbent hormonal milieu is more 'anabolic' than another; however, we see no evidence that this thesis is correct. Our work, using best practice methods and direct assessments, is state-of-the-art in this field and should provide robust guidance for follow-up research.

It is also notable that the classification of E2 as a potentially anabolic hormone during the follicular phase of the menstrual cycle is often extrapolated from OVX animal models, some of which have demonstrated that OVX impairs skeletal muscle growth, maintenance or repair of muscle tissue (Bar et al., 1988; Enns & Tiidus, 2008; Enns et al., 2008), but this conclusion ignores the fact that OVX does not exclusively affect E2, but also depletes P4, LH and follicle-stimulating hormone (Coyle-Asbil et al., 2023) and is model of replete and completely deplete E2 status, and in no way recapiluates menstrual cycle fluctuations in hormones. Given the interaction between the ovarian hormones, it is difficult to confidently identify a purely oestrogen-specific mechanism that would show an effect on skeletal muscle. Even acknowledging that ovarian hormone depletion is problematic, the OVX model does not translate directly to humans. The complete ablation of hormonal activity is dissimilar to the regular oscillatory changes in ovarian hormone during the menstrual cycle and notablly the gradual, non-linear decline in hormones during menopause.

Future research should be aimed at exploring the influence of endogenous and exogenous ovarian hormone fluctuations on longitudinal changes in muscle size and strength with a more rigorous methodological approach than has been employed in previous trials. Although Sakamaki-Sunaga et al. (2016) found no effect of menstrual cycle phase-specific training on hypertrophy, Sung et al. (2014) reported that training during the follicular phase was superior (leading to an ∼1.8 mm

greater increase in the summed diameter of the rectus femoris, vastus intermedius and vastus lateralis in the limb trained during the follicular phase) to training during the luteal phase for muscle strength and hypertrophy. Wikström-Frisén et al. (2017) also concluded that follicular phase-based training was superior to luteal phase-based training and, surprisingly, was superior to training throughout the full cycle for gains in leg lean mass. However, Wikström-Frisén et al. (2017) included both naturally menstruating participants and individuals on oral contraceptives in their sample, and no study used a high-quality method to identify cycle and phase length. Sung et al. (2014) stated that all their study participants had a 28-day cycle length and ovulated on day 14, as assessed with basal body temperature. Given the inter-individual variability in cycle length and ovulation timing (D'Souza et al., 2023) and the prevalence of such a cycle length in the current study, we estimate that these investigators (Sung et al., 2014) would have had to screen ∼250 females to recruit their sample of females with the menstrual features they claim (i.e. 28 day cycle length and all ovulating on day 14). In the absence of high-quality data (Colenso-Semple et al., 2023; D'Souza et al., 2023) showing a significant influence of menstrual cycle phase on exercise-induced adaptations or performance, pre-menopausal female participants should not be excluded from exercise science studies solely on concerns about their hormonal status as a potential confounding variable, at least in studies involving muscle protein anabolism and likely other outcomes.

Using best practice approaches to characterise menstrual cycle phase, we conclude that fluctuations in ovarian hormones do not affect muscle anabolism or myofibrillar proteolysis in response to resistance exercise. Our data therefore show that there is no ostensible advantage to planning exercise to emphasise resistance exercise-induced anabolism in one menstrual phase over the other in normally menstruating females.

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## Additional information

### Data availability statement

Data are available upon reasonable request from the corresponding author.

### Competing interests

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### Author contributions

All authors have approved the final version of the manuscript submitted for publication and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship and all those who qualify for authorship are listed.

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### Keywords

exercise, human muscle, menstrual cycle, protein metabolism

## Supporting information

Additional supporting information can be found online in the Supporting Information section at the end of the HTML view of the article. Supporting information files available:

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