Understanding the female athlete: molecular mechanisms underpinning menstrual phase differences in exercise metabolism

Authors:

Tanja Oosthuyse[1,2]*, Juliette A. Strauss[3], Anthony C. Hackney[4]

Affiliations:

[1] School of Physiology, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa

[2] Division of Physiological Sciences, Department of Human Biology, University of Cape Town, Cape Town, South Africa

[3] Research Institute for Sport & Exercise Sciences, Liverpool John Moores University, Liverpool, UK

[4] Department of Exercise and Sport Science, Department of Nutrition, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

***Corresponding author:** Tanja Oosthuyse

Division of Physiological Sciences, University of Cape Town,

Sports Science Institute of South Africa, Boundary Road,

Newlands 7700, Cape Town, South Africa

Email: oosthuyse@polka.co.za; Tanja.Oosthuyse@wits.ac.za

Tel: +27 83 400 9088

ORCIDs: Tanja Oosthuyse ORCID: 0000-0002-4065-4506

Juliette Strauss ORCID: 0000-0001-7175-2494

Anthony Hackney ORCID: 0000-0002-7437-6225

Abstract

Research should equitably reflect responses in both men and women. Including women in research, however, necessitates an understanding of the ovarian hormones and menstrual phase variations in both cellular and systems physiology. This review outlines recent advances in the multiplicity of ovarian hormone molecular signalling that now elucidates the mechanisms for menstrual phase variability in exercise metabolism. The molecular structure of the prominent endogenous estrogen, 17-β-estradiol (E2), is itself bioactive in stabilising plasma membranes and quenching free-radicals and both E2 and progesterone (P4) promote the expression of endogenous antioxidant enzymes attenuating exercise-induced muscle damage in certain menstrual phases. E2 and P4 bind nuclear hormone receptors and membrane-bound receptors to regulate gene expression directly or indirectly through multiple paths, which importantly includes cross-regulated expression of their own receptors. In addition, activation of membrane-bound receptors regulates kinases that feed into multiple cellular pathways to bring about rapid cellular responses. Furthermore, receptor-type dependent outcomes result in tissue-specific responses with variable inter-organ dependence. Careful analysis of these signaling pathways now explains the menstrual phase-specific differences in hepatic glucose production, whole body plasma glucose uptake, tissue specific lipid storage and fat oxidation, and protein catabolism during exercise, and post exercise muscle satellite cell recruitment, activation, and proliferation. This review is expertly presented in an integrative manner, from a cellular level to whole-body outcomes, providing a causative effect that is supported by meta-analyses, where appropriate, for quantitative actuality. Importantly, evidence-based relevance for training and performance in female athletes and areas warranting further research exploration are highlighted.

Keywords: Premenopausal women, Eumenorrhea, Estrogen and progesterone signaling, Exercise metabolism, Exercise-induced muscle damage

Abbreviations

ACC	acetyl-CoA carboxylase
Akt	protein kinase B
AMPK	5'AMP-activated protein kinase
AP-1	activator protein 1
BCOAD	branched-chain 2-oxo-acid dehydrogenase
cAMP	cyclic-AMP
CD36	fatty acid translocase
CEBPA	CCAAT/enhancer-binding protein alpha
CK	creatine kinase
CPT-1	carnitine palmitoyl transferase-1
CREB	cAMP-response element binding protein
DOMS	delayed onset muscle soreness
EIMD	exercise-induced muscle damage
E2	17-β-estradiol
EF	early follicular
ER	estrogen receptor
ERE	estrogen response elements
ERK	extracellular signal-related kinases
FABPc	fatty acid binding protein

FAS	fatty acid synthase
FATmax	exercise intensity at maximal fat oxidation
FATP	fatty acid transport protein
FOXO1	forkhead box protein O1
FSH	follicle-stimulating hormone
FSR	fractional protein synthetic rate
G6Pc	glucose-6-phosphotase
GLUT4	glucose transporter 4
GnRH	gonadotropin-releasing hormone
GPER	G-protein estrogen receptor
β-HAD	β-hydroxyacyl-CoA dehydrogenase
IMTG	intramuscular triglyceride
IRS	insulin receptor substrate
LCFA	long chain fatty acid
LF	late follicular
LH	luteinizing hormone
LPL	lipoprotein lipase
MCAD	medium-chain fatty acyl-CoA dehydrogenase
MF	mid-follicular
ML	mid-luteal
mPR	membrane progestin receptors
mtGPAT	mitochondrial glycerol-3-phosphate acyl transferase
mtTFA	mitochondrial transcription factor A
Myo-D	myogenic differentiation factor D

72	NFκB	nuclear factor kappa-B
73	NRF-1	nuclear respiratory factor-1
74	OC	oral contraceptive
75	OVX	ovariectomized
76	p38MAPK	p38 mitogen activated protein kinase
77	P4	progesterone
78	Pax7	paired box homeotic gene 7
79	PDK-1	phosphoinositide-dependent protein kinase-1
80	PDK-4	pyruvate dehydrogenase kinase-4
81	PEPCK	phosphenolpyruvate-carboxykinase
82	PGRMC	progesterone G-protein receptor membrane complex
83	PI3K	phosphoinositide-3-kinase
84	PPAR	peroxisome proliferation activator receptor
85	PR	progesterone receptor
86	PRE	progesterone response elements
87	Ra	rate of appearance
88	Rac1	Ras-related C3 botulinum toxin substrate 1
89	Rd	rate of disappearance
90	RDI	recommended daily intake
91	*Slc2a4*	solute carrier family 2 member 4
92	SOD	superoxide dismutase
93	SP-1	specific protein 1
94	SREBP1c	sterol regulatory element-binding protein 1c
95	T3	triiodothyronine

96	T4	thyroxine
97	TBC1D	TBC1Domain family member
98	TCA	tricarboxylic acid
99	TFPα	trifunctional protein-alpha
100		

Introduction

Since the early 1980s researchers have observed menstrual phase differences in the metabolic response to exercise that can influence exercise capacity or performance (for example, Jurkowski et al. 1981; Lavoie et al. 1987; Nicklas et al 1989). Subsequent research evaluated the influence of the menstrual phase or purposeful manipulation of the ovarian hormones on various aspects of carbohydrate, fat, and protein metabolism during exercise to isolate the underlying modulations and the metabolic state that is required for these changes to be notable (for reviews see, Oosthuyse and Bosch, 2010; Hackney 2021; Boisseau and Isacco 2022). More recently, a clearer understanding of the molecular pathways and cellular interactions of the ovarian hormones has helped to elucidate the mechanisms that bring about the menstrual phase-specific differences. Understanding the ovarian hormone metabolic signaling mechanisms will improve future research design striving to optimise training and performance in female athletes. This review aims to describe the menstrual phase ovarian hormonal effects on exercise metabolism while highlighting the molecular (signaling) mechanisms. With that objective, this review: (i) defines the menstrual phases; (ii) presents the molecular structure of estrogen as a mechanistic factor instrumental in regulating exercise-induced muscle damage (EIMD); (iii) introduces the receptor-activated signaling pathways of estrogen and progesterone; (iv) reviews the effects of the menstrual cycle on carbohydrate, fat and protein metabolism during exercise and the regulatory signaling mechanisms for each (including meta-analyses where appropriate to describe the magnitude of effect on the oxidation or turnover of the various endogenous energy sources); and (v) introduces the effect of the menstrual cycle on muscle regeneration and strength. Additionally, practical relevance for female athletes and areas warranting further research exploration are highlighted.

Defining the menstrual phases

The ovarian status of women is multiplexed and requires methodological considerations when conducting research in women (Elliott-Sale et al. 2021). The current review focuses on premenopausal, eumenorrheic women who experience natural, ovulatory menstrual cycles. The reproductive physiology of a eumenorrheic (normal, regular length, ovulatory) menstrual cycle is comprehensively described by Mihm et al. (2011), where the predictable rise and fall in the circulating ovarian hormones, estrogen, and progesterone, are explained in the context of the feedback loops of the anterior pituitary hormones, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which in-turn respond to hypothalamic gonadotropin-releasing hormone (GnRH), to co-ordinate follicle development and regression. This is a worthwhile read for researchers who aim to include female participants in studies. In this current review, we only consider the predictable flux of estrogen and progesterone across a typical eumenorrheic cycle, which is partitioned into the following phases: early follicular (EF), mid-follicular (MF), late follicular (LF), ovulation, early luteal, mid-luteal (ML) and late luteal phases (Fig. 1). It is noteworthy that while a large proportion of premenopausal women experience menstrual cycles of ~28 days, the length of a eumenorrheic cycle can vary between 21 to 35 days (Elliott-Sale et al. 2021). Mihm et al. (2011) explain that women with the more typical shorter cycle length follow a 2-wave follicular cycle while women with longer cycle lengths follow a 3-wave follicular cycle, with respect to follicle development that culminates in the selection of a dominant follicle. Thus, the longer menstrual cycle in some eumenorrheic women is owing to a longer follicular phase whereas the luteal phase is more consistently ~14 days (Mihm et al. 2011). This means that the time to ovulation is variable but after ovulation has occurred, menses can be expected ~2 weeks later. Janse de Jonge et al. (2019) has outlined what are deemed acceptable methods for predicting and verifying menstrual phase and ovulation. Accordingly, menstrual phase comparative studies

are required to verify the menstrual cycle phase by reporting measured estrogen and progesterone concentrations. To-this-end, only menstrual phase comparative studies that reported estrogen and/or progesterone concentrations are included in this review. Stricker et al. (2006) measured serum ovarian hormone concentrations in daily samples taken from 20 eumenorrheic women and these median values can be used to guide researchers in defining the acceptable concentration ranges when verifying menstrual phases in female participants (Table 1). Most comparative studies compare EF (or MF) and ML phases because these phases represent the extremes of low and high ovarian hormones, respectively. The LF phase is occasionally included in studies, presenting an ideal time to evaluate the effect of estrogen without progesterone.

insert Figure 1 and Table 1 near here

Estrogen modulates EIMD by virtue of its molecular structure

Estrogen and progesterone (P4) are synthesized primarily in the ovaries by canonical steroidogenesis with cholesterol as the primary precursor, and to a lesser extent in other tissues that express the enzyme, aromatase and on availability of a C19 steroid precursor (Barakat et al. 2016). The pathway outlining human steroidogenesis has been schematically constructed as a useful reference showing the key enzymes and intersecting paths to produce firstly the progestogens, which progresses to produce either the mineralocorticoids or androgens and finally the estrogens (see Häggström and Richfield 2014). The three main endogenous estrogens include: estrone (E1), 17-β-estradiol (E2), and estriol (E3), with a fourth estrogen, estetrol (E4) only present during pregnancy, where the assigned numeral defines the number of hydroxyl groups. E2 is the most potent and prominent form that is assessed in most menstrual phase comparative studies and is used as the default when

175 referring to endogenous estrogen in this review. E2 is metabolized by hydroxylation to form
176 less potent catechol estrogen metabolites. The hydroxylated E2 is then deactivated by
177 methylation via catechol-O-methyltransferase. In this way, E2 competes with catecholamines
178 for deactivation and can thereby increase the half-life of catecholamine action. Or
179 alternatively, E2 is oxidized to estrone (E1) before being deactivated by conjugation with
180 sulfate or glucuronide groups (Fuentes and Silveyra 2019). All active estrogens, including
181 some estrogen metabolites and estrogen mimetics or dietary phytoestrogens are biologically
182 active by binding to various estrogen receptors (Gregorio et al. 2021).

183 ***insert Figure 2 near here***

184

185 The molecular structure of E2 (Fig. 2a) also accounts for certain bioactive effects
186 independent of ligand-receptor activated signaling. The phenolic structure and hydroxyl
187 groups of E2 affords it strong lipophilic and antioxidant potency that are key features in E2
188 protection of phospholipid membrane stability and integrity against lipid peroxidation
189 (Kendall and Eston 2002). E2 is proposed to act like tocopherol in free radical quenching
190 where the hydroxyl groups function as proton donors to reduce and quench free radicals. As
191 with cholesterol, the phenolic structure of E2 allows it to intercalate in plasma membrane
192 bilayers to improve membrane stability while halting lipid peroxidation chain reactions
193 (Kendall and Eston 2002) (Fig. 2a). These features are particularly relevant for endurance
194 exercise metabolism, where prolonged high rates of oxidative energy production are
195 associated with free radical production that compromises the function and integrity of the
196 sarcolemma resulting in skeletal muscle inflammation and damage. To-this-end, the post
197 exercise leakage of muscle proteins, such as creatine kinase (CK), into circulation is largely
198 explained by compromised sarcolemma function because of exercise-associated oxidative

damage and membrane instability (Tiidus 2003; Enns and Tiidus 2010). For this reason, the change in serum CK activity following exercise is a marker of sarcolemma stability. In a series of early studies, Tiidus and colleagues found that E2 treatment versus placebo in ovariectomized (OVX) rodents attenuated the increase in serum CK activity after exercise and this protection increased markedly with the duration of estrogen therapy (reviewed by Tiidus 2003; Enns and Tiidus 2010). Similarly, estrogen treatment reduced the influx of neutrophils into muscle and the activity of the protease, calpain in muscle following exercise. Tiidus (2003) described the sequence for estrogen's protection as follows: the sarcolemma is stabilized by estrogen, which results in less calcium leaking into the cytosol and thus less calpain activation. Reduced calpain activity results in less expression of chemoattractant peptides which explains the decrease in neutrophil infiltration and overall reduced muscle damage; encompassing the primary pro-inflammatory phase of the muscle damage/repair process (Peake et al. 2017). Therefore, it is not surprising that sex-differences in resting serum CK activity and post exercise CK response have been reported with lower resting CK levels in women and reduced post exercise CK response (Sewright et al. 2008; Luk et al. 2021; Kumagai et al. 2022) or quicker return to resting levels in women than men (Oosthuyse and Bosch 2017). Furthermore, given that population variability in the genotypes of alleles within genes can translate into some differences in the functionality of the expressed protein, it is interesting that men having a TT genotype polymorphism in the gene for the E2 producing enzyme, aromatase has higher E2 concentrations and reduced CK response after an ultramarathon than men with CT/CC genotype (Kumagai et al. 2022). Moreover, recent evidence showed women to have a muted intramuscular cytokine response to muscle damaging exercise compared with men (Luk et al. 2021) with previous evidence of reduced infiltration of granulocytes into the muscle in women versus men (Stupka et al. 2001). Interestingly, while the serum CK and delayed onset muscle soreness (DOMS) recovery

times are associated in men, they are unrelated in women (Sewright et al. 2008; Oosthuyse and Bosch, 2017). Nociceptive sensitivity may be influenced by menstrual phase, causing DOMS to poorly reflect restoration of sarcolemma function and muscle repair in women (Oosthuyse and Bosch 2017).

Considering that E2's plasma membrane protection depends partly on the molecular structure of E2 and thus only appreciated when E2 is present and possibly more so with elevated concentrations, it is likely menstrual phase specific. Accordingly, the increase in serum CK activity 24 h and 48 h after 20 min of downhill running was negatively correlated with serum E2 concentrations in eumenorrheic women who participated in either their EF, LF or ML phase (Oosthuyse and Bosch 2017). Likewise, serum CK activity was consistently lower 24 h and 72 h after 60-90 min of level running at 70%VO_2peak in the ML compared with MF phase (Williams et al. 2015; Hackney et al. 2019); with a negative correlation between the increase in serum CK activity and serum E2 concentrations (Williams et al. 2015) and a decreased serum interleukin-6 response at all time points after exercise in the ML versus MF phase (Hackney et al. 2019). A recent meta-analysis attempted to quantify the effect of the menstrual phase on markers of EIMD by including all prior studies in females and found no clear menstrual phase effect on the serum CK response (Romero-Parra et al. 2021). However, the inclusion of studies that only considered a single menstrual phase or did not provide adequate verification of the menstrual phase by means of ovarian hormone concentrations suggests the outcome of that meta-analysis should be graded as low certainty and interpreted with caution. Such a between-study comparison of effect lacks sensitivity owing to differing severities of exercise protocols that would undoubtedly influence the CK response variably independent of menstrual phase. Furthermore, when an unaccustomed mechanical stress is extremely high, such as during maximal force eccentric exercise, the severity of structural

damage to muscle fibers might not be rescued by the effect of E2 to preserve membrane function. In that case, a similar large serum CK response may occur irrespective of menstrual phase (for example see, Funaki et al. 2022). Therefore, menstrual phase differences in EIMD due to E2 ability to stabilize membranes and protect against lipid peroxidation may be expected following endurance-type exercise (for example, Williams et al. 2015; Oosthuyse and Bosch, 2017; Hackney et al. 2019), which imposes lower muscle forces over prolonged periods requiring a high flux of oxidative phosphorylation.

The cellular protective effect of E2 persists even in the presence of the estrogen receptor inhibitor, tamoxifen (Paroo et al. 2002), supporting the functional role of the molecular structure of E2 to be instrumental in conferring these benefits independent of certain typical ligand-receptor signaling. However, E2 signaling is complex and multifaceted with several direct and indirect genomic and non-genomic paths of action that are introduced here-in. Indeed, E2 signaling does increase the expression of the antioxidant defence enzymes, superoxide dismutase (SOD) (Strehlow et al. 2003) and glutathione peroxidase (Viña et al. 2008). And therefore, the antioxidant role of E2 appears to be a sum of its role as both a structural antioxidant and genomic activator. Agreeably, a recent study quantified the presence of reactive oxygen metabolites and tested the antioxidant potential in blood samples taken before and after high intensity intermittent exercise in women during their EF and ML phases (Matsuda et al. 2020). They reported a 10% increase in circulating reactive oxygen metabolites in the EF, but not ML phase after exercise, together with a tendency for a greater increase in antioxidant potential after exercise in ML than EF phase. Albeit the ML phase is associated with an increase in both E2 and P4.

P4, unlike E2, is not a structural antioxidant and is in fact susceptible to reduction at the ketone groups or carbon double bond (positioned between C4 and C5; from where the abbreviation, P4 derives its numeral) (Fig. 2b). P4, however, has been shown to reduce free radical damage by increasing the expression and activity of the antioxidant defence enzymes, SOD, glutathione peroxidase, and glutathione reductase via P4 receptor signaling (Hernández-Rabaza et al. 2019). For this reason, the synergistic effect of E2 and P4 in the ML phase to reduce oxidative stress supports evidence for the ML phase to be better than the EF phase for exercise that risks EIMD.

Although beyond the intended scope of this review, it must be noted that the condition of oral contraceptive (OC) use may alter these responses as discussed previously (Oosthuyse and Bosch 2017). Furthermore, recent accumulation of evidence supports a higher level of oxidative stress and inflammatory markers such as C-reactive protein in female athletes taking combined OCs compared with non-OC users (Cauci et al. 2017, 2021; Larsen et al. 2020; Quinn et al. 2021).

E2 and P4 receptor-activated signaling

Ligand-receptor activated signaling pathways of E2 and P4 are mechanistically similar and have been reviewed in detail (for E2 signaling see: Foryst-Ludwig and Kintscher 2010; Yaşar et al. 2016; Fuentes and Silveyra 2019; Puglisi et al. 2019) (for P4 signaling see: Garg et al. 2017; Gonzalez et al. 2020; Medina-Laver et al. 2021). Signaling occurs either via the classical path of binding nuclear hormone receptors or the more recently discovered alternate route of binding membrane receptors (Fig. 3), as detailed below.

Nuclear hormone receptors

Both E2 and P4 influence metabolism by binding estrogen and progesterone nuclear hormone receptors that occur as E2 receptor-alpha (ERα) and E2 receptor-beta (ERβ) or P4 receptor-A (PR-A) and P4 receptor-B (PR-B) isoforms, respectively. Receptor isoform specificity is relevant because the various receptor isoforms are linked to differing metabolic outcomes (Foryst-Ludwig and Kintscher, 2010; Mauvais-Jarvis et al. 2013; Gregorio et al. 2021). E2 and P4 bind to the respective isoforms with equal affinity and the net outcome is, therefore, dependent on the tissue-specific isoform abundance (Yaşar et al. 2016). E2 and P4 diffuse through plasma membranes to bind their respective nuclear hormone receptors in the cytosol. On binding, they translocate to the nucleus where the ligand bound receptors assemble typically as dimer complexes to act as ligand-activated transcription factors and bind estrogen response elements (ERE) or progesterone response elements (PRE), respectively, in the promoter regions of target genes to activate or repress gene transcription (Yaşar et al. 2016; Puglisi et al. 2019). E2 and P4 bound to their cognate nuclear hormone receptors can also regulate expression of genes that do not have ERE or PRE in the promoter regions by binding to other DNA-bound transcription factors of those respective target genes (Foryst-Ludwig and Kintscher, 2010; Yaşar et al. 2016; Puglisi et al. 2019; Gonzalez, 2020).

Mitochondrial DNA also includes EREs and E2-ER binding on mitochondrial DNA has been shown to enhance the expression of a protein subunit of ATP synthase and mitochondrial manganese SOD expression (Yaşar et al. 2016). Furthermore, nuclear E2-ER binding activates nuclear respiratory factor-1 (NRF-1) expression, which subsequently activates nuclear expression of mitochondrial DNA transcription factors such as mitochondrial

transcription factor A (mtTFA) (Yaşar et al. 2016; Ventura-Clapier et al. 2019). In this way, E2 promotes mitochondrial biogenesis and capacity for oxidative phosphorylation. Both ERα and ERβ isoforms have been identified in mitochondria as well as membrane-bound E2 receptors (Ventura-Clapier et al. 2019).

Membrane-bound receptors

E2 and P4 induce rapid signaling in seconds or minutes by binding membrane-bound receptors coupled to G proteins that activate various kinases, namely: phosphoinositide-3-kinase (PI3K)/Akt, extracellular signal-related kinases (ERK), p38 mitogen activated protein kinase (p38MAPK), and others, and thereby E2 and P4 act in a non-genomic manner to phosphorylate and regulate the activity of other cellular proteins (Puglisi et al. 2019; Gonzalez, 2020). The activated kinases can also phosphorylate nuclear bound E2 or P4 nuclear hormone receptors that suffices to activate or repress expression of target genes having ERE or PRE in their promoter regions without the need for direct E2 or P4 ligand binding (Yaşar et al. 2016; Puglisi et al. 2019). Similarly, the activated kinases can phosphorylate various other nuclear bound transcription factors to indirectly regulate gene expression of other target genes lacking ERE or PRE and without any interaction of E2 and P4 nuclear hormone receptors (Yaşar et al. 2016; Puglisi et al. 2019). In this way, E2 and P4 can impose indirect genomic regulation when binding their membrane-bound receptors. Furthermore, E2 regulates calcium ion channels and sarcoplasmic/endoplasmic reticular calcium transporters to modulate cytosolic and mitochondrial calcium content and associated signaling cascades (Mahmoodzadeh and Dworatzek, 2019; Puglisi et al. 2019). E2 is also functional in the phosphorylation of 5'AMP-activated protein kinase (AMPK) and rapidly increases its activity within less than 10 min of administering E2 *in vitro* or *in situ* (D'Eon et

al. 2005; D'Eon et al. 2008; Rogers et al. 2009; Gorres et al. 2011), which in skeletal muscle occurs selectively via ERα but not ERβ (Gorres et al. 2011). Conversely, in vascular endothelial cells, E2 binding ERβ (but not ERα) increased intracellular calcium content and activation of calcium calmodulin-dependent protein kinase kinase-β to phosphorylate AMPK, independent of the more typical upstream AMPK kinase, liver kinase B1 (Yang and Wang, 2015); thus, emphasizing tissue-specific signalling.

It is proposed that the membrane-bound receptors can include: (1) the classical E2 and P4 nuclear hormone receptors that are in this instance membrane-bound and (2) further receptor family classes referred to as G-protein estrogen receptors (GPER) (Puglisi et al. 2019) or progesterone G-protein receptor membrane complex (PGRMC) and membrane progestin receptors (mPR) (Garg et al. 2017; Gonzalez et al. 2020; Medina-Laver et al. 2021). Recent evidence in adipocytes suggests that of the E2 classical nuclear hormone receptor isoforms, only ERα translocate to the plasma membrane in the presence of E2, whereas ERβ remain nuclear bound (Campello et al. 2017; Gregorio et al. 2021). For this reason, it is likely that E2 membrane-receptor signaling, and rapid non-genomic effects occur mostly via specifically E2-ERα or E2-GPER interactions and not E2-ERβ; albeit possibly tissue specific. Studies in mice report expression of ERα > GPER > ERβ in both soleus (type 1 fibers) and extensor digitorum longus (type 2 fibers) muscles (Baltgalvis et al. 2010) and expression of ERα > ERβ in not only skeletal muscle but also liver and adipose (Ribas et al. 2010).

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P4 represses ER expression

Evidence exists for E2 and P4 cross-interactions that are often antagonistic (Campbell and Febbraio, 2001, 2002) with the net outcome in the luteal phase dependent on the relative serum concentrations. We now know that P4 antagonism of E2 can in part or in whole be explained by P4 inhibition or repression of ER expression and protein content (Jayaraman and Pike 2009; Ekenros et al. 2017). In an *in vitro* study in neurons, E2 promoted ERα and ERβ expression and P4 downregulated this effect rapidly within 15 min and the level of repression increased with greater incubation time (Jayaraman and Pike 2009). P4 repression was maximal at typical ML concentrations of 30 nmol/L (Jayaraman and Pike 2009). Furthermore, skeletal muscle ER and PR expression and protein content have been compared between EF, ovulation, and ML phases in sedentary eumenorrheic women, where ER mRNA and protein content was found to be lowest in the ML phase (Ekenros et al. 2017). The order of skeletal muscle ERα mRNA content occurred as: EF > ovulation > ML that coincided with typical serum P4 concentrations that were lowest in EF, modestly higher at ovulation, and substantially higher in ML. Accordingly, ERα protein content was greater in the EF phase than ML phase. In that study, the modest serum E2 concentrations reported during the ovulation sampling period (200 pmol/L) might suggest that sampling may have occurred in the recent post ovulation phase when E2 concentrations fall off rapidly and it would have been insightful had sampling been conducted in the LF phase coincident to peak E2 concentrations. Moreover, PR protein content was found to be greater in the ML than both at ovulation and EF phase suggesting that P4 promotes PR abundance, which conversely does not appear to be antagonised by the concomitant increase in E2 in the ML phase (Ekenros et al. 2017) and in fact PR expression is activated by E2-ER signaling (Medina-Laver et al. 2021). These mechanistic uncovering's of P4's modulation of E2 signaling supports early suggestions to consider the relative concentrations of E2 and P4 in the ML phase when interpreting outcomes (D'Eon et al. 2002; Horton et al. 2002).

Mechanisms behind menstrual cycle effects on carbohydrate metabolism

Overview of exercise-stimulated plasma glucose uptake

Our understanding of insulin signaling, and exercise-stimulated plasma glucose uptake has advanced. The basic framework of the insulin signaling pathway includes insulin binding to membrane-bound insulin receptor tyrosine kinase that phosphorylates insulin receptor substrate (IRS) that then binds phosphoinositide-3-kinase (PI3K) resulting in the generation of PI-3,4,5-triphosphate to activate phosphoinositide-dependent protein kinase-1 (PDK-1) and initiates two signaling pathways. Firstly, the PI3K pathway phosphorylates and activates serine/threonine protein kinase B also known as Akt, which phosphorylates the Rab-GTPase activating proteins, TBC1Domain family member1 (TBC1D1) or TBC1D4 (also known as AS160), and thereby relieves inhibition on Rab-GTPase that then facilitates translocation of GLUT4 storage vesicles to the plasma membrane (Sakamoto and Holman 2008). Secondly, PI3K activates Ras-related C3 botulinum toxin substrate 1 (Rac1), a Rho-family GTPase, that regulates scaffold proteins and remodels the actin cytoskeleton to facilitate GLUT4 translocation and therefore plasma glucose uptake (Yue et al. 2020). Exercise-stimulated plasma glucose uptake acts independently of insulin and the current viewpoint is that it is partly reliant on AMPK to directly phosphorylate TBC1D1/4 (de Wendt et al. 2021); although evidence suggests that while AMPK is essential for increasing glucose uptake after exercise, it is not essential during exercise (Kjøbsted et al. 2019; McConell, 2020). Instead, exercise-stimulated glucose uptake is promoted by mechanical stress or stretch that activates integrin receptors and focal adhesions, or dystrophin-glycoprotein complexes, which then promote Rac1 activity and GLUT4 translocation (Sylow et al. 2017; Flores-Opazo et al. 2020) (Fig. 4). However, it is also possible that the AMPK and Rac1 pathways driving

exercise-stimulated glucose uptake are inter-related (Yue et al. 2020; de Wendt et al. 2021). A recent viewpoint suggests that the increase in glucose uptake with exercise is not only dependent on GLUT4 translocation but also possibly an increase in GLUT4 activity (Richter, 2021). Furthermore, during exercise it can be assumed that all of the plasma glucose taken up is shuttled into glycolysis and oxidation (Sylow et al. 2017) and therefore the measured rate of plasma glucose uptake is often used as a surrogate for the rate of plasma glucose utilization during exercise.

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E2 and P4 influence of plasma glucose uptake

E2 and P4 signaling feed into insulin signaling pathways (Wada et al. 2010; Mauvais-Jarvis et al. 2013) and in addition E2 influences GLUT4 expression (reviewed by Gregorio et al. 2021) to affect glucose uptake (Fig. 4), as outlined in the sections that follow.

E2 regulation of GLUT4 expression

While there is no ERE in the promoter of the gene (solute carrier family 2 member 4, *Slc2a4*) encoding GLUT4, E2-ER exerts genomic effects by binding various *Slc2a4* transcription factors (Gregorio et al. 2021). E2 influence is dependent on the ER isoform where E2-ERβ trans-represses and E2-ERα transactivates GLUT4 expression (Campello et al. 2012; Barreto-Andrade et al. 2018). For example, E2-ERα binds the inflammation-stimulated transcription factor, nuclear factor kappa-B (NFκB), and thereby blocks its repression of GLUT4 expression (Campello et al. 2012); E2-ERα binds specific protein 1 (SP-1) on the promoter of *Slc2a4* causing transactivation of GLUT4 expression (Barreto-Andrade et al.

2018); E2-ERα binds CCAAT/enhancer-binding protein alpha (CEBPA) to transactivate GLUT4 expression and also directly increases the expression and content of CEBPA and promotes its translocation to the nucleus (Fatima et al. 2019). The mechanism explaining ERβ trans-repression of GLUT4 expression is not clear but may be via inhibition of SP-1 (Gregorio et al. 2021).

P4 alone did not change adipocyte GLUT4 expression *in vitro* (Wada et al. 2010) but did reduce GLUT4 protein content in adipose tissue and skeletal muscle of OVX rodents compared with sham-operated rodents (Campbell and Febbraio, 2002). In fact, GLUT4 content was suppressed even when P4 was administered with E2 and P4-inhibition was only overcome with a supra-physiological E2 dose (Campbell and Febbraio, 2002). Thus, P4 effect on GLUT4 expression or protein content may be indirect possibly in part due to P4-repression of ERα expression. Accordingly, skeletal muscle GLUT4 mRNA content was greater in women in their follicular phase compared with men and was increased 2-fold in men after 8 days of oral E2 supplementation that mimicked LF phase serum E2 concentrations (946 pmol/L) (Fu et al. 2009). Furthermore, a comparison between menstrual phases revealed a trend for 2.2-fold greater skeletal muscle GLUT4 mRNA content during the follicular than luteal phase (Fu et al. 2009), where the luteal phase was characterised by modest serum E2 (203 pmol/L) and P4 (6 nmol/L) concentrations more typical of the early or late luteal phase and possibly represented a P4 dominant effect.

E2 and P4 regulation of GLUT4 translocation

E2 binding specifically ERα at the plasma membrane induces PI3K/Akt phosphorylation and promotes GLUT4 translocation independent of insulin (Barros et al. 2006; Rogers et al. 2009;

463 Gorres et al. 2011; Campello et al. 2017). Thus, E2-ERα can initiate both the classical
464 TBC1D1/4 pathway and Rac1 pathway. In addition, E2-ERα phosphorylation of AMPK
465 initiates AMPK phosphorylation of TBC1D1/4 (Rogers et al. 2009; Gorres et al. 2011).

466

467 In this way, E2 via ERα increases GLUT4 expression and GLUT4 translocation to the
468 plasma membrane and is thus expected to enhance exercise-stimulated plasma glucose
469 uptake. Accordingly, exercise-stimulated glucose uptake is 1.4-fold greater in the skeletal
470 muscle of females compared with male mice (Kang et al. 2021). Furthermore, endurance
471 training increases ERα abundance in skeletal muscle (Lemoine et al. 2002; Wiik et al. 2005).
472 Therefore, we might expect E2's effect on plasma glucose uptake to be most notable in
473 endurance trained women.

474

475 However, P4 decreases insulin-stimulated glucose uptake by decreasing the abundance of
476 IRS and interfering with signalling distal to IRS and thereby reducing GLUT4 translocation
477 in adipocytes (Wada et al. 2010); albeit possibly tissue-specific considering a P4 positive
478 effect on glucose uptake and GLUT4 expression in neurons (Wu et al. 2019). Accordingly,
479 exercise-stimulated plasma glucose uptake in skeletal muscle was reduced by OVX in rodents
480 and restored after 15 days of treatment with E2 alone, but not with P4 alone (Campbell and
481 Febbraio 2002). In fact, P4 when combined with E2, prevented the increase in exercise-
482 stimulated glucose uptake that could be overcome with a supraphysiological dose of E2
483 (Campbell and Febbraio 2002). For these reasons, the relative increase in E2 and P4 in the
484 ML phase will determine the net outcome for potential menstrual phase changes specifically
485 to skeletal muscle exercise-stimulated glucose uptake and GLUT4 content, with the LF phase

likely presenting the most favourable time for this E2-dominant effect to be realized and certainly warrants investigation in eumenorrheic athletes.

However, despite convincing evidence for the tissue-specific effects of E2 to enhance exercise-stimulated glucose uptake, this can be constrained by ovarian hormone influences on whole-body energy homeostasis via multi-tissue governance, as uncovered in the sections that follow.

Whole-body plasma glucose uptake during exercise

A substantial number of studies have measured whole-body plasma glucose uptake during exercise either during different menstrual phases in fed (Campbell et al. 2001; Suh et al. 2002) or fasted (Campbell et al. 2001; Zderic et al. 2001; Horton et al. 2002; Devries et al. 2006) eumenorrheic women, or after purposeful controlled manipulation of ovarian hormone concentrations in non-OC users (D'Eon et al. 2002), or in amenorrheic women (Ruby et al. 1997) and men (Carter et al. 2001; Devries et al. 2005) on short-term E2 treatment. We have combined the findings from these studies in a summative meta-analysis; where the mean (and SD) rate of plasma glucose uptake measured in μmol/kg·min in the EF phase/placebo trial is compared with the ML phase/hormone supplemented trial in each study to derive a mean difference and effect size as the standardised mean difference using Review Manager (RevMan version 5.4.1) (Fig. 5a).

While most studies included in the meta-analysis did in fact apply a within-participant crossover design, results were only presented as between-individual means (and SD) for each

phase or condition instead of the mean within-individual change. For this reason, the findings presented reflect independent between-group and not dependent within-group outcomes and the meta-analysis outcome likely underestimates the true effect, adopting a conservative approach. In all studies, stable isotopic-tracer methodology was applied by constant intravenous infusion of [6,6-^{2}H]glucose to attain steady-state enrichment before plasma glucose rate of appearance (Ra) and rate of disappearance (Rd) could be derived from validated equations based on the principles of tracer dilution (Kim et al. 2016), where glucose Rd represents the rate of whole-body plasma glucose uptake. The meta-analysis outcome shows, first, in agreement with the strong antagonistic effect of P4 as discussed above, the ML phase is associated with a decrease in the rate of plasma glucose uptake compared with the EF phase, with a moderate effect during fasted exercise and a small non-significant effect when exercising fed (Fig. 5a). Thus, demonstrating that ovarian hormone effects are more notable during periods of metabolic stress or a high demand for endogenous substrates. Second, while we may expect conditions of short-term E2 treatment alone in men or amenorrheic women to increase the rate of plasma glucose uptake during exercise, instead studies also report a significant decrease with E2 treatment compared with placebo (Fig. 5a). This seemingly discrepant finding may be a result of men and amenorrheic women, who experience chronic low E2 concentrations, also having low ERα content in skeletal muscle and greater relative ERβ content as is typical in E2 deficient conditions, such as menopause (Foryst-Luwig and Kintscher, 2010; Park et al. 2017). However, in the study by D'Eon et al. (2002), where ovarian hormones in eumenorrheic women were suppressed with a GnRH antagonist followed by treatment with E2 alone or combined E2 and P4, the rate of plasma glucose uptake during exercise also tended to be reduced with E2 treatment (or E2 plus P4) compared with ovarian suppression. Thus, suggesting that despite prior evidence for tissue-specific effects in skeletal muscle for E2 to increase exercise-stimulated glucose uptake, on a

whole-body level E2 reduces the rate of plasma glucose uptake during exercise. The current meta-analysis demonstrates an overall outcome for the ML phase/E2 treatment compared with the EF phase/placebo treatment to produce a small negative effect of -4.18 μmol/kg·min on the rate of plasma glucose uptake during exercise lasting 50-120 min at 50-70%VO_2max (Fig. 5a), that equates to -2.7 g/h of glucose taken up for 60 kg women. Such a magnitude of effect may appear to have little physiological relevance over the duration of exercise that has been tested, but it remains to be determined whether longer duration exercise produces larger and more meaningful menstrual phase effects.

insert Figure 5 near here

Moreover, oral contraceptive therapy for 4 months has been found to reduce the rate of plasma glucose uptake during exercise by up to 20% (-8.44 μmol/kg·min) even when exercising fed (3 h postprandial) compared with before-OC use in eumenorrheic women (Suh et al. 2003). It may be that OC potency is greater than the naturally cycling endogenous hormones resulting in a larger effect that becomes notable even with less metabolic stress. Such a decrease in the rate of plasma glucose uptake with OC use versus non-OC use equates to -5.5 g/h of glucose taken up for 60 kg women, an amount that represents 5% of total carbohydrate oxidation (reported as 1.75 g/min or 105 g/h) during exercise at 65%VO_2max (Suh et al. 2003). Some suggest that the oral route of delivery of hormonal contraceptives imposes high hepatic exposure via the portal vein, which could magnify ovarian hormone receptor binding and downstream signaling responses in hepatocytes (Magkos et al. 2022).

As is typically expected, the rate of plasma glucose uptake (Rd) approximates the rate of glucose appearance in plasma (Ra) under the rigorous homeostatic regulation of blood

glucose concentration (Kim et al. 2016). Accordingly, menstrual phase or hormonal conditional outcomes on the rate of plasma glucose appearance during exercise mimics the rate of plasma glucose uptake and is similarly presented in a summative meta-analysis (Fig. 5b). During fasted exercise, plasma glucose Ra largely reflects hepatic glucose production derived from hepatic glycogenolysis and gluconeogenesis. Considering evidence for E2 to increase skeletal muscle-specific glucose Rd capacity during exercise, and yet whole-body plasma glucose Rd is reduced in the presence of elevated E2 (and P4) specifically during fasted exercise, it is likely that plasma glucose Rd in ML (and possibly LF) phase is limited by plasma glucose Ra and the influence of E2 (and P4) on hepatic glucose production.

Ovarian hormone influence on hepatic glucose production

In the 1970's, a study on rodents reported that 21-days of E2 and P4 treatment suppressed gluconeogenesis on provision of isotopic-glucogenic precursors at rest (Matute and Kalkhoff, 1973). More recently, the respective molecular signaling mechanisms have been uncovered. A study using OVX mice with E2 treatment alone or in combination with an Akt inhibitor or ERα inhibitor found that E2 suppresses gluconeogenesis and thus hepatic glucose production (Yan et al. 2019). E2 acts by binding ERα at the plasma membrane to induce PI3K and Akt phosphorylation, where Akt, in turn, phosphorylates forkhead box protein O1 (FOXO1) resulting in its nuclear export and degradation and thereby prevents FOXO1 binding to the promoters of the key gluconeogenic enzymes, glucose-6-phosphotase (G6Pc) and phosphenolpyruvate-carboxykinase (PEPCK) (Yan et al. 2019). In this way, E2 like insulin, represses the expression of these gluconeogenic enzymes (Fig. 4).

Conversely, the P4 effect on gluconeogenesis is variable depending on carbohydrate availability (Lee et al. 2020). With carbohydrate provision, P4 enhances insulin secretion to suppress gluconeogenesis in healthy insulin-sensitive states, as seen in male mice with P4 treatment (Lee et al. 2020). Alternatively, in a glucose-depleted condition, as may occur during fasted exercise, P4 in hepatocytes increases glucose production by increasing the expression of the key gluconeogenic enzymes (G6Pc and PEPCK). An *in vitro* model using hepatocytes shows, P4 binds P4 receptor membrane complex 1 (PGRMC1) to activate adenylate cyclase and induce cyclic-AMP (cAMP) that in turn activates protein kinase A, which phosphorylates the transcription factor, cAMP-response element binding protein (CREB) to promote expression of G6P and PEPCK (Lee et al. 2020) (Fig. 4). However, considering plasma glucose Ra during fasted exercise is reduced in the ML phase, we can assume that in eumenorrheic women, E2 suppression of hepatic glucose production predominates.

Overriding hepatic glucose production annuls the decrease in plasma glucose uptake during exercise in the ML phase

Only one previous study compared plasma glucose Rd when a carbohydrate supplement (60 g/h of glucose) was ingested during 2 h of exercise at 70%VO_2max during the EF and ML phases (Campbell et al. 2001). Ingesting carbohydrate compared with placebo increased glucose Ra similarly in the EF and ML phase (Fig. 5b), sourced mainly from absorption of the ingested supplement with hepatic glucose production contributing minimally. With a surplus plasma glucose supply, the rate of plasma glucose uptake increased similarly in both EF and ML phase (Fig. 5a), implying that the combined increase of E2 and P4 in the ML phase failed to produce an expected E2-augmented exercise-stimulated glucose Rd.

Furthermore, a recent study overcame the limitation of E2 or P4 influence on gluconeogenesis and hepatic glucose production by implementing an intravenous 10 mM hyperglycemic clamp during 90 min of exercise at 60%VO_2max in the follicular phase and luteal phase and the rate of glucose uptake was inferred from the rate of glucose infusion required to maintain the clamp (Hulton et al. 2021). The menstrual phase timing of the luteal phase trials did not coincide with the ML phase in all participants and possibly rather represents early luteal or late luteal phase in some subjects as serum E2 concentration increasing from a mean ~100 pmol/L in follicular phase to ~200 pmol/L in luteal phase and serum P4 concentration attaining only a mean of 14 nmol/L in luteal phase with P4 increasing above the 16 nmol/L threshold to confirm an ovulatory cycle in only 2 of the 7 participants. Notwithstanding, this study also found that the rate of plasma glucose uptake during exercise remains unchanged between follicular and luteal phases when plasma glucose availability is not limiting (attaining a high peak glucose Rd of 131μmol/kg·min that equates to 1.4 g/min for a 60 kg women) (Hulton et al. 2021). These findings remain to be confirmed by further studies that encompass LF and ML phases with the provision of oral multiple-transportable carbohydrates during exercise at maximal tolerable doses (Trommelen et al. 2017).

It is well established that the availability of carbohydrate as an energy source is important for promoting the best performances in moderate-high intensity exercise (Burke and Hawley 2018). Accordingly, overnight-fasted high-intensity time trial performance performed after a 2 h preload, was compromised in ML phase compared with the EF phase, coincident to reduced plasma glucose Ra (or hepatic glucose production) and glucose Rd (Campbell et al. 2001). Interestingly, when overnight-fasted premenopausal women ingested a glucogenic

substrate in the form of rapidly absorbed casein protein hydrolysate versus placebo during 80 min of moderate-hard exercise in a crossover design matched for the menstrual phase, metabolism shifted in favour of greater carbohydrate oxidation that coincided with a small increase in distance covered and total work done during the final 20 min time trial (Oosthuyse et al. 2021). Thus, the restriction of E2 (and P4) on gluconeogenesis during the ML and possibly LF phases can have negative implications for exercise performance under certain conditions of prolonged exercise requiring high intensity efforts without adequate carbohydrate supplementation. For this reason, the ability to store muscle and liver glycogen is also important.

Influence of the ovarian hormones on glycogen storage and use

Studies have found a greater ability to replete muscle glycogen stores after glycogen depleting exercise in the ML phase compared with MF phase (Nicklas et al. 1989; Hackney 1990) and when following a normal mixed diet including ~5 g/kg carbohydrate (MF: 575 mmol/kg d.w. vs. ML: 728 mmol/kg d.w.) (McLay et al. 2007). Although this menstrual phase difference disappears when following a high carbohydrate-loading diet for 3 days (~8 g/kg carbohydrate) (McLay et al. 2007). Similarly, when ingesting a high carbohydrate dose (1.2 g/kg per hour) during the immediate 5 h recovery period after glycogen depleting exercise, a similar muscle glycogen repletion is achieved in EF, LF, and ML phases (Matsuda et al. 2022b). E2 increases glycogen synthase activity (Beckett et al. 2002) possibly by acting via membrane-bound ERα to stimulate PI3K/Akt signaling to phosphorylate glycogen synthase kinase-3α thereby releasing its inhibition on glycogen synthase activity (Yan et al. 2019) and by increasing glycogen synthase expression as seen in men after 8 days of oral E2 supplementation (Fu et al. 2009). However, it may be less relevant when following a high

carbohydrate-rich diet and during acute recovery when ingesting a high-dose carbohydrate supplement that results in an already maximal insulin-stimulated signaling response.

Muscle glycogen use during moderate-intensity endurance exercise (60-70% VO_2max) based on analysis of muscle biopsies has been reported to be 24% and 25% less in the ML versus EF phase in two independent studies, respectively, coincident to greater fat oxidation (Hackney 1999; Devries et al. 2006) where glycogen use was inversely correlated with E2 concentration in the ML phase (Hackney 1999). Likewise, the rate of glycogen use at 60%VO_2max, estimated from the difference between the rate of total carbohydrate oxidation and tracer measured plasma glucose Rd, was 24% and 28% less in eumenorrheic women on controlled E2 supplements mimicking the LF phase also coincident to greater fat oxidation compared with ovarian hormone suppression and compared with combined E2 and P4 supplements, respectively (D'Eon et al. 2002). Interestingly, the combined E2 and P4 supplements caused a supraphysiological increase in P4 possibly reflecting a P4-dominant condition (D'Eon et al. 2002). Thus, the glycogen sparing effect of E2 may occur secondary to greater fat oxidation during moderate-intensity endurance exercise and can be countered by P4 and hence is dependent on the relative increase in E2 and P4 (Hackney et al. 2022). Conversely, high-intensity exercise necessitates carbohydrate-sourced energy production, and consequently, E2 suppression of hepatic gluconeogenesis might explain recent findings for greater muscle glycogen use, measured by ^{13}C-magnetic resonance spectroscopy, during overnight-fasted high-intensity intermittent exercise to exhaustion in the LF than EF phase (Matsuda et al. 2022a). However, the greater muscle glycogen use during exercise in the LF phase in this study was found to be significant despite no menstrual phase differences in the absolute starting or ending muscle glycogen concentration (Matsuda et al. 2022a).

Mechanisms behind menstrual cycle effects on fat metabolism

E2 signaling increases fat oxidation capacity

Campbell and Febbraio (2001) reported that 15-days of E2 treatment in OVX rodents increased the maximal activity of enzymes regulating long chain fatty acid (LCFA) oxidation in skeletal muscle, namely carnitine palmitoyl transferase-1 (CPT-1) that governs LCFA transport across the mitochondrial membrane and β-hydroxyacyl-CoA dehydrogenase (β-HAD), the third enzyme in beta oxidation. Coincident treatment with P4 blocked E2's effect on these enzymes, where P4 antagonism could be prevented by a supraphysiological dose of E2 (Campbell and Febbraio 2001).

Subsequently, a collection of studies largely from three different laboratories have uncovered the molecular signaling pathway whereby E2 promotes fat oxidation (Fig. 6). The cumulative findings from these studies that employed either an OVX-rodent model, *in vitro* cell incubation, men on E2 supplements or sex-differences have been reviewed in detail elsewhere (Tarnopolsky 2008; Oosthuyse and Bosch, 2012). Briefly, in skeletal muscle, E2 bound ERα activates gene transcription of the nuclear hormone receptors, peroxisome proliferation activator receptor-alpha (PPARα) and PPARδ either directly by binding ERE in the gene promoter regions or indirectly via membrane bound ERα to induce phosphorylation of AMPK (Campbell et al. 2003; D'Eon et al. 2005, 2008; Fu et al. 2009; Salehzadeh et al. 2011). PPARα and PPARδ activate the expression of downstream targets (some of which may also be under direct genomic regulation of E2) to increase the protein content of plasma membrane (fatty acid transport protein (FATP), fatty acid translocase (CD36)), cytosolic

(fatty acid binding protein (FABPc)) or mitochondrial (CPT-1) LCFA transporters and mitochondrial beta oxidation enzymes (medium-chain fatty acyl-CoA dehydrogenase (MCAD); β-HAD; beta oxidation enzyme complex, trifunctional protein-alpha (TFPα)) and the glycolytic down-regulator, pyruvate dehydrogenase kinase-4 (PDK4) to direct substrate utilization away from carbohydrate and in favour of fat oxidation (Campbell et al. 2003; D'Eon et al. 2005; Fu et al. 2009; Maher et al. 2010a, 2010b). Recent evidence suggests that the Rab-GTPase activating proteins, TBC1D1/4 that regulate GLUT4 translocation are also involved in regulating LCFA transporter content and trafficking to the plasma membrane and thus LCFA oxidation (Benninghoff et al. 2020; Mikłosz et al. 2021). TBC1D1/4 maintains the Rab GTPase proteins in an inactive GDP-bound state and this inhibition is released upon phosphorylation of TBC1D1/4 by its upstream effectors (Akt and AMPK), which then allows Rab-GTP binding and hydrolysis to facilitate transporter translocation. In this way, investigations are needed to establish whether E2 via membrane bound ERα phosphorylation of PI3K/Akt and AMPK may possibly also contribute to LCFA transporter translocation and increased LCFA uptake in skeletal muscle.

In addition, E2 increases lipid availability specifically in skeletal muscle by firstly, increasing the expression of the transcription factor, sterol regulatory element-binding protein 1c (SREBP1c), which activates gene expression of downstream targets of skeletal muscle intramuscular triglyceride (IMTG) storage, namely, mitochondrial glycerol-3-phosphate acyl transferase (mtGPAT) for re-esterification and acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) for lipogenesis (Fu et al. 2009). Secondly, E2 via increased PPARδ increases expression and content of skeletal muscle localized lipoprotein lipase (LPL) to support hydrolysis of plasma triglyceride for skeletal muscle LCFA uptake (D'Eon et al. 2005). Thirdly, E2 promotes a redistribution of LCFA away from adipose tissue and toward skeletal

muscle by reducing adipose tissue localized LPL activity to reduce triglyceride uptake in adipose (Ellis et al. 1994; Homma et al. 2000) and by reducing adipose-specific lipid storage via tissue-specific inhibition of SREBP1c expression in adipose and liver (D'Eon et al. 2005) and by promoting adipose triglyceride lipolysis by enhancing beta-adrenergic sensitivity to epinephrine (Benoit et al. 1982), reducing antilipolytic alpha$_2$-adrenergic responsiveness (Schmidt et al. 2014) and increasing perilipin protein content (albeit without identifying subtype specificity) (D'Eon et al. 2005). The role of different perilipin protein isoforms in the regulation of triglyceride storage and lipolysis in various tissues and sex-differences in perilipin content have been reviewed (MacPherson and Peters 2015). The actions of perilipins are important for preventing the accumulation of lipid oxidation intermediates and accordingly, perilipin actions differ from the basal state to the hormone-stimulated lipolytic state (MacPherson and Peters 2015).

P4 when administered in combination with E2 in OVX rodents modulates E2 effect to increase PPARα and PDK4 (Campbell et al. 2003). Without further studies investigating the direct mechanistic effect of P4 on lipid transport or metabolic pathways, currently P4 modulation of E2 actions can possibly only be attributed to P4's repression of ER expression and protein content (Jayaraman and Pike 2009; Ekenros et al. 2017).

insert Figure 6 near here

Menstrual cycle effect on whole-body fat oxidation

Taking the prior points into account, it is not surprising that sex-differences in the rate of whole-body fat oxidation are frequently reported particularly during fasted exercise (for

748 Reviews see, Devries, 2016; Boisseau and Isacco 2022; Cano et al. 2022). Surprisingly,
749 however, menstrual phase differences are less consistent, with only some reporting a greater
750 rate of fat oxidation in the LF (Hackney et al. 1991) and ML phases compared with EF phase
751 during fasted exercise (for example, Hackney et al. 1994, 1999; Campbell et al. 2001; Zderic
752 et al. 2001; Willett et al. 2021). However, E2 promotion of fat oxidation during exercise is
753 confirmed with men on E2 supplements (Hamadeh et al. 2005) and, as already mentioned, in
754 premenopausal women on controlled E2 treatment versus ovarian suppression or combined
755 E2 and supraphysiological P4 treatment (D'Eon et al. 2002). In fact, the change in the rate of
756 fat oxidation during exercise from EF to ML phase has been found to correlate positively
757 with the change in the E2/P4 ratio (Hackney et al. 2022). Moreover, in one study, a greater
758 rate of fat and lower carbohydrate oxidation in the ML versus MF phase was significant at
759 low (35%VO_2max) and moderate (60%VO_2max) exercise intensities but not significant at a
760 higher intensity (75%VO_2max) (Hackney et al. 1994) possibly where a higher rate of
761 carbohydrate utilization becomes obligatory. Therefore, it seems that a notable shift in whole-
762 body substrate utilisation with the menstrual phase is only evident: (i) during fasted exercise;
763 or (ii) mainly when exercise duration places a sufficiently high demand on endogenous
764 energy stores; and (iii) possibly only at or below the exercise intensity marking the metabolic
765 threshold after which carbohydrate utilization predominates; and (iv) as such is dependent on
766 a high relative concentration of E2 to P4 in the ML phase.

767

768 For example, the exercise intensity eliciting maximal fat oxidation (FATmax) and the peak
769 fat oxidation rate is not different between MF, LF and ML phases when measured in a
770 standard 3-min step incremental test to exhaustion (Frandsen et al. 2020). This is not
771 surprising because the 3-min steady-state steps are too short to pressurize energy metabolism
772 and fuel reserves sufficiently to reveal potential menstrual phase differences that may

possibly become evident should exercise duration at each workload be substantially longer. Conversely, with a similar test protocol, sex-differences in peak fat oxidation rate expressed relative to fat-free mass were evident, being greater in women than men (Chrzanowski et al. 2021) with this sex-difference evident only in women <45 years but not >45 years (Frandsen et al. 2021) that likely differentiates premenopausal versus peri- or post-menopausal life stages and thus cyclical E2 availability versus chronic E2-deficiency as the underlier.

Fat oxidation source: IMTG or plasma LCFA-derived

Sex-differences in fat oxidation may be partly owing to greater IMTG content in women (because of a greater number of lipid droplets and not greater lipid droplet size) and greater localization of lipid droplets near mitochondria in women (Devries et al. 2007; Beaudry and Devries 2019), together with reports for superior mitochondrial functional capacity in women compared with men matched for training status (Cardinale et al. 2018; Montero et al. 2018), that are E2-attributable perturbations according to signaling mechanisms already discussed. Albeit early variations in methods of IMTG quantification (i.e., biochemical extraction, immunofluorescence microscopy or proton magnetic resonance spectroscopy) have resulted in an unclear consensus for sex-differences in IMTG use during exercise (Steffensen et al. 2002; Roepstorff et al. 2002, 2006; White et al. 2003; Devries et al. 2007). Furthermore, whether the cyclical fall and rise in E2 with the respective menstrual phases notably alters IMTG content, sub-cellular location and use during exercise in eumenorrheic women has not been evaluated and certainly warrants investigation. Improvements in histological staining techniques has advanced our understanding of IMTG myofibrillar-localization and association with perilipin proteins, IMTG utilization during exercise and resynthesis in

796 recovery (Jevons et al. 2020; Strauss et al. 2020) but are yet to be applied in menstrual phase
797 comparative studies.

798

799 Most interestingly, IMTG use during exercise is not reduced by ingestion of carbohydrate
800 supplements in men (Fell et al. 2021) with confirmation needed for the same response in
801 women. However, IMTG use is dependent on exercise duration and intensity, and varies
802 inversely with plasma fatty acid availability and use (Lundsgaard et al. 2018). At high
803 exercise intensities, fat oxidation is limited at the point of LCFA entry into the mitochondria
804 by free carnitine availability where the excess acetyl-CoA produced by the glycolytic
805 enzyme, pyruvate dehydrogenase is buffered by carnitine acetyltransferase resulting in the
806 entrapment of free carnitine as acetylcarnitine to support the maintenance of a high-demand
807 glycolytic rate (Lundsgaard et al. 2018). This limits the activity of CPT1 where free carnitine
808 is required for the conversion of long chain fatty acyl-CoA to fatty-acyl-carnitine for
809 mitochondrial uptake. Thus, unlike the assumption of plasma glucose uptake, not all plasma
810 LCFA taken up into skeletal muscle during exercise is necessarily oxidized. Excess LCFA
811 uptake is shuttled into re-esterification and IMTG storage. During low-moderate intensity
812 exercise, with lower glycolytic flux, IMTG roughly accounts for 30% of fat oxidation
813 (Lundsgaard et al. 2018) with plasma-derived LCFA largely from adipose tissue lipolysis
814 accounting for 60% of fat oxidation, in men (Lundsgaard et al. 2018) and women (Jacobs et
815 al. 2005).

816

817 Only two menstrual phase comparative studies have measured the rate of lipolysis and the
818 rate of plasma LCFA uptake during exercise by intravenous infusion of the stable-isotopic
819 tracers, [1,1,2,3,3-^{2}H]glycerol and [1-^{13}C]palmitate, respectively. The first study reports no

820 change between EF and ML phases in glycerol kinetics (Casazza et al 2004) and no change in
821 the rate of plasma LCFA appearance, uptake, or oxidation (Jacobs et al. 2005) at either
822 45%VO_2max or 65%VO_2max. However, the small sample size (n = 5) and high variability in
823 especially plasma LCFA kinetics and oxidation in the ML phase encourages further follow up
824 studies. Furthermore, in this former study the women participated fed (3 h postprandial) and
825 exercise lasted only 60 min, which might also partly explain the lack of differences. The
826 second study included a larger sample of women (n = 11) who were overnight-fasted and
827 exercised for 90 min at 50%VO_2max, but some menstrual phase timing errors resulted in a
828 few missing data points and data being evaluated using a less sensitive unpaired groups
829 design (Horton et al. 2006). Nonetheless, this latter study also reports no differences between
830 EF, MF and ML phases in the rate of plasma glycerol or LCFA appearance and uptake during
831 exercise but was without measurement of plasma LCFA oxidation. The findings from these
832 two studies have been combined in a summative meta-analysis that may encourage further
833 studies to investigate menstrual cycle effects on particularly plasma LCFA oxidation and flux
834 during exercise (Fig. 7a). This meta-analysis, however, does present a clear outcome for no
835 effect on plasma glycerol Ra and Rd, reflecting an unchanged whole-body lipolytic rate
836 during exercise between EF and ML phases in these studies (Fig. 7b). In addition, E2
837 treatment in amenorrheic women for 6 days (Ruby et al. 1997) or men for 8 days (Carter et al.
838 2001) failed to alter plasma glycerol kinetics during exercise further supporting the likelihood
839 of no notable ovarian hormone effect on whole-body lipolysis between menstrual phases or
840 with short-term E2 treatment. Of note, the above studies also all failed to find a difference in
841 total whole-body fat oxidation during exercise between menstrual phases or ovarian
842 conditions. It could be that the serum E2 concentration recorded in the ML phase in these
843 studies (E2: 311 pmol/L, P4: 34.7 nmol/L and E2: 393 pmol/L, P4 36.6 nmol/L, respectively)
844 was not high enough for ovarian or menstrual phase effects on fat metabolism to become

apparent; compared with studies that have reported a shift in substrate utilisation towards greater fat oxidation in the ML phase (for example, E2: 519 pmol/L, P4 47.9 nmol/L (Willet et al. 2021) where the change in fat oxidation from EF phase correlated with the change in the E/P ratio (Hackney 2022). Others have suggested that the latter studies where E2 treatment was administered (Ruby et al. 1997; Carter et al. 2001) were likely underpowered for the given variability considering that a clear shift towards greater whole-body fat oxidation during exercise was noted in a larger sample of 12 males receiving similar dose oral E2 supplements (Hamadeh et al. 2005). It would be insightful if, in future studies, plasma glycerol and LCFA kinetics and plasma LCFA oxidation were evaluated during exercise where a coincident shift in whole-body metabolism towards greater fat oxidation in the LF or ML compared with EF phase is evident.

insert Figure 7 near here

Conversely, OC use for 4 months increased plasma cortisol concentration and whole-body lipolysis during exercise, as indicated by stable-tracer measured plasma glycerol Ra that increased by more than 20%, compared with before OC use in eumenorrheic women (Casazza et al. 2004). However, the greater rate of lipolysis was coupled with a greater rate of FFA re-esterification and less plasma FFA being oxidized during exercise, measured by plasma [1-^{13}C]palmitate tracer kinetics, after 4 months of OC use compared with before OC use and hence indicates a futile cycle of substrate mobilization (Jacobs et al. 2005). Yet again, this demonstrates that OC use influences exercise metabolism differentially compared with naturally cycling endogenous ovarian hormones experienced by eumenorrheic women, where differences also extend to other physiological parameters, such as basal inflammatory

status (Cauci et al. 2017, 2021; Larsen et al. 2020; Quinn et al. 2021) and bone turnover (Allaway et al. 2020).

Notably, the technical difficulties of administering LCFA tracers by intravenous infusion could be a deterring factor to explain the shortage of such menstrual phase comparative studies. The hydrophobic nature of LCFA tracers necessitates constitution with 5% human serum albumin as a carrier protein that could pose ethical risk limitations. Furthermore, use of LCFA carbon-13 tracers to measure plasma LCFA oxidation from expired [^{13}C]-carbon dioxide enrichment requires correction for possible retention of the carbon-13 label in the exchange or transamination reactions that occur with tricarboxylic acid (TCA) cycle intermediates (Sidossis et al. 1995a, 1995b). To this end, the acetate correction factor was proposed that must be derived concurrently with plasma LCFA-tracer measured oxidation or in an additional identical exercise trial with the constant infusion of carbon-13 labelled sodium acetate (Sidossis et al. 1995b). When the acetate correction factor was derived during 90 min of cycling at 60%VO_2max in a small sample (n = 5) of eumenorrheic women, 2-3 h post prandial, during the EF, LF, and ML phases it was found to be modestly lower in the ML (0.57) than EF (0.59) phase despite no difference in whole-body substrate utilisation (Oosthuyse et al. 2003). While application of the correction factor would increase absolute plasma LCFA oxidation rate notably, the small difference between menstrual phases would only equate to a 6% error in the measured change in plasma LCFA oxidation between menstrual phases. Nevertheless, the greater carbon-label fixation in TCA cycle exchange reactions in the ML phase does suggest an increased flux in transamination reactions in the ML phase, which may be suggestive of greater protein catabolism during exercise.

Mechanisms behind menstrual cycle effects on protein metabolism

Increased protein catabolism in the ML phase

Lamont et al. (1987) were first to report greater total urea nitrogen excretion (a sum of urine and sweat urea nitrogen) in the ML versus EF phase in recreationally active women measured over 3 days that included a 60 min cycling exercise on day 1, indicating greater protein catabolism in the ML phase. According to these findings, protein requirements per day in EF were notably less than in the ML phase (1.05 g/kg vs. 1.36 g/kg per day, respectively). In agreement, amino acid isotopic tracer studies in eumenorrheic women have consistently reported a greater rate of leucine or phenylalanine oxidation during the ML versus EF phase when fasted (Lariviere et al. 1994; Toth et al. 2006) and fed (Kriengsinyos et al. 2004). These studies, however, were measured at rest and did not include exercise. The summative findings from these studies are presented in a meta-analysis showing a moderate effect of the menstrual cycle phase on plasma amino acid oxidation rate (Fig. 8a) but no clear effect on plasma amino acid flux (where at rest Ra = Rd) (Fig. 8b). Moreover, lysine dietary requirements based on the indicator amino acid oxidation method was found to be higher in the ML than EF phase (37.7 mg/kg versus 35.0 mg/kg per day, respectively) (Kriengsinyos et al. 2004). In this study carbon-labelled lysine was ingested in increasing doses on separate occasions over several consecutive menstrual cycles in each participant and the rate of lysine oxidation measured from the carbon-label enrichment in expired carbon dioxide that increases disproportionately on reaching an ingestion dose in excess of the requirement to support protein synthesis. Considering that lysine oxidation was maintained at a higher rate at all ingestion doses in ML versus EF phase, suggests the higher lysine requirement in ML phase is due to higher amino acid catabolism (Kriengsinyos et al. 2004).

insert Figure 8 near here

Greater protein catabolism during the ML phase appears to be a P4 dominant effect because when men received oral E2 supplements (2 mg/day) for 8 days to mimic plasma E2 concentration during the LF phase (876 pmol/L), leucine oxidation was notably decreased at rest (PL: 28 ± 2 vs E2: 23 ± 2 μmol/kg·h) and during 90 min of exercise at 65%VO_2max (PL: 62 ± 5 vs E2: 53 ± 4 μmol/kg·h) (Hamadeh et al. 2005). This resulted in a less negative protein balance after E2 supplements compared with placebo. However, leucine flux and the rate of protein synthesis were not altered by E2 supplements.

The mechanism whereby P4 upregulates and E2 downregulates protein catabolism remains undefined. E2 does not appear to alter the expression of certain enzymes involved in amino acid catabolism. For example, in the same men just noted receiving 8 days of oral E2 supplementation to mimic the LF phase (Hamadeh et al. 2005), E2 supplementation did not alter skeletal muscle expression of the amino acid transamination enzyme, aspartate aminotransferase, or the rate-limiting enzyme for branched-chain amino acid oxidation, branched-chain 2-oxo-acid dehydrogenase (BCOAD), or its negative regulator, BCOAD kinase (Fu et al. 2009). However, skeletal muscle BCOAD kinase mRNA content was 1.2-1.3-fold greater in women than men at rest (Fu et al. 2009), which in its role as a negative regulator of BCOAD could reduce branch-chain amino acid oxidation and thereby partly explain the frequently reported lower rate of protein catabolism in women than men (Tarnopolsky 2008). In that same study, skeletal muscle mRNA content of BCOAD kinase in women was not different between the follicular (E2, 125 pmol/L; P4, 2.0 nmol/L) and luteal (E2, 203 pmol/L; P4, 6.0 nmol/L) phase, but the interpretation of this finding is limited by the modest differences in ovarian hormone concentrations between phases suggesting that the

measurement in the luteal phase may have corresponded with the late luteal phase (Fu et al. 2009). Future studies should investigate the effect of P4 on these gene targets. Furthermore, whether the ovarian hormones regulate skeletal muscle protein content or activity of transamination and branched-chain amino acid oxidation enzymes remains to be determined. Alternatively, some suggest P4 upregulation and E2 downregulation of thyroid function and availability of free triiodothyronine (T3) (Ben-Rafael et al. 1987; Torre et al. 2020) is causative of the greater protein catabolism in the luteal phase (Lariviere et al. 1994). Resting plasma free T3 concentration was found to be higher coincident to a greater leucine oxidation rate in the ML versus EF phase in one study (Lariviere et al. 1994). P4 binds PR within the thyroid gland to promote T3/thyroxine (T4) secretion and reduce its binding to the plasma protein, thyroid-binding globulin to maximise free T3 availability (Torre et al. 2020; Kaminski et al. 2021) thereby accounting for the higher resting metabolic rate (Benton et al. 2020) and thus possibly protein catabolism reported in the luteal phase compared with follicular phase.

The estimated average daily requirement of protein for female endurance athletes derived from 3-day nitrogen balance measurements in two studies conducted in the MF phase in female cyclists training 13 and 11 h/week is between 1.28 and 1.63 g/kg per day with high interindividual variability (95%CI: 0.7-1.85 and 1.1-3.8 g/kg per day, respectively) (Rowlands and Wadsworth, 2011; Houltham and Rowlands et al. 2014). Based on these findings, recommended daily intake (RDI) of protein for female endurance athletes has been suggested as 1.6-2.0 g/kg per day, to allow for 12% interindividual variability (Mercer et al. 2020) but does not include menstrual phase specificity, which warrants further validation.

No menstrual phase effect on muscle protein synthesis

Only one study in eumenorrheic women has quantified the rate of muscle protein synthesis between menstrual phases. In this study, unilateral leg exercise was performed with 1-legged kicks and 1-leg acting as a resting control in the EF and ML phases (Miller et al. 2006). Twenty-four hours later, fractional protein synthetic rate (FSR) was measured from a 4 h constant infusion of [1-^{13}C]leucine followed by a muscle biopsy to measure the percentage uptake of the label into muscle myofibrillar protein in each leg. While FSR was higher in the exercised leg 24h-post exercise compared with the resting leg, there was no menstrual phase differences in resting (EF: 0.053±0.009%/h vs ML: 0.055±0.013%/h) or 24h-post exercise (EF: 0.131±0.018%/h vs ML: 0.134±0.018%/h) FSR (Miller et al. 2006).

Nevertheless E2-deficiency, as occurs in menopause, is known to promote muscle atrophy by increased autophagic signalling through FOXO3 that is supressed by E2 replacement, where E2 replacement increases muscle mass and strength further when combined with exercise training possibly by regulation of satellite cell activity (Hansen, 2018). It may be that while menstrual phase does not influence resting or recovery muscle protein synthesis in eumenorrheic women, E2 could enhance exercise-stimulated anabolism by the activation and proliferation of muscle satellite cells following exercise in a menstrual phase specific manner, as presented below.

Menstrual phase influences muscle regeneration and strengthening

Enns and Tiidus (2008) have clearly demonstrated these effects in OVX rodents receiving E2 replacement by showing a greater increase in paired box homeotic gene 7 (Pax7)-positive

cells, a marker of satellite cell number, a greater increase in myogenic differentiation factor D (Myo-D)-positive fibers indicating satellite cell activation, and a greater increase in DNA uptake of bromo-deoxyuridine suggesting satellite cell proliferation after downhill running with E2 treatment versus placebo. This group went on to show that these E2 effects on satellite cells were ERα dependent (Thomas et al. 2010). In fact, E2-ER increases Myo-D expression indirectly by binding the transcription factor, activator protein 1 (AP-1) and thereby prevents AP-1 repressive action on Myo-D expression (Pedraza-Alva et al. 2009).

Interestingly, E2 influence over the satellite cell response to EIMD is more noticeable during the MF compared with ML phase as shown by Haines et al. (2018) after unilateral eccentric knee extensor exercise. These authors found ERα muscle content and the increase in ER-DNA binding after exercise to be greater in the MF than ML phase (Haines et al. 2018). Accordingly, the increase in expression of Myo-D, reflecting satellite cell activation, appeared greater and cyclin D1 reflecting mitotic cycling was significantly greater in the MF than ML phase after eccentric exercise (Haines et al. 2018). In this study, serum E2 concentration, although significantly higher in the ML phase (410 pmol/L) was also substantially elevated in the MF phase (325 pmol/L) and consequently muscle E2 concentration was not different between MF and ML phases. Thus, the noted difference in ER and satellite cell activity occurred independent of a difference in muscle E2 concentration and suggests that P4 in the ML phase suppressed E2 promotion of satellite cell differentiation and muscle regeneration after EIMD, possibly by downregulation of ER expression and protein content as already discussed (Jayaraman and Pike 2009; Ekenros et al. 2017). Although it is also true that EIMD is consistently less in ML than EF phase as detailed earlier and as such decreased plasma membrane disruption and EIMD in ML may also contribute to a reduced need for satellite cell activation and muscle repair in ML than EF phase.

Consequently, a study that performed unilateral leg resistance training with 1-leg trained only in the follicular phase and the other leg trained only in the luteal phase for 3 menstrual cycles found greater muscle strength gain and muscle diameter in the follicular phase trained-leg than the luteal phase trained-leg (Sung et al. 2014). This occurred coincident to an increase in the nuclei-to-fiber ratio in muscle from the follicular phase leg only, that is said to suggest notable satellite cell recruitment in the muscle trained in the follicular phase (Sung et al. 2014). Similar findings support greater muscle strength gain from high frequency (4-5 d/week) leg resistance training when performed specifically during the follicular phase rather than luteal phase (Reis et al. 1995; Wikström-Frisén et al. 2017), with only a single report of no difference in outcomes after follicular phase-based versus luteal phase-based arm resistance training at a lower frequency (1-3 d/week) (Sakamaki-Sunaga et al. 2016).

Therefore, muscle regeneration and strengthening are mostly greater during the follicular phase when E2 is present alone (even in low-moderate concentrations) without P4.

Conclusion and perspectives

The molecular mechanisms of E2 and P4 are diverse and underpin menstrual phase differences in exercise metabolism. At this stage research has uncovered firstly, that E2 phenolic structure stabilizes membranes and quenches free radicals, while genomic effects of E2 and P4 increase antioxidant defence causing the ML phase to be the best time for unaccustomed endurance exercise or low intensity resistance loads owing to a modulated EIMD response. Secondly, E2 promotion of skeletal muscle glucose uptake is not realized in the LF or ML phase owing to E2 dominant repression of hepatic gluconeogenic enzyme expression and plasma glucose availability that can compromise high intensity exercise

efforts in carbohydrate-depleted states. For this reason, carbohydrate supplements during exercise are imperative in these menstrual phases and future studies should explore menstrual phase effects when ingesting multiple-transportable carbohydrates at recommended doses during exercise, as well as variability in efficacy of ingesting exogenous glucogenic sources, such as rapidly absorbed peptides. Thirdly, E2 signaling to increase glycogen synthase activity and thereby promote increased glycogen storage in the ML (or possibly LF) phase at normal daily carbohydrate intakes should be considered for recovery or when planning pre-event nutrition that will necessitate higher carbohydrate-loading intakes to equalize glycogen stores in the EF (or MF) phase. Fourthly, E2 signaling favors skeletal muscle fat uptake, storage, and utilisation but exercise intensity and carbohydrate availability are the dominant regulators of substrate partitioning, such that a menstrual phase increase in whole-body fat oxidation is only realized in the ML (or LF) phase under carbohydrate-restricted exercise and with a sufficiently high E2:P4 ratio. Currently, insufficient studies have evaluated for menstrual phase differences in the lipid-derived source, namely plasma LCFA or IMTG, during exercise. Fifthly, protein catabolism has consistently been shown to be greater in the luteal phase at rest and the possibility for RDI of protein to be menstrual phase specific should be explored. In particular, assessment of menstrual phase differences in protein catabolism during exercise should be investigated and the possibility of recommending ingestion of rapidly absorbed peptides during exercise or more rigorous protein recovery supplements in the ML should be considered. Moreover, the molecular signaling mechanism explaining the P4 dominant effect promoting protein catabolism should be investigated. Lastly, athletes including adjunct strength training should periodize these training sessions in the follicular phase to benefit from greater muscle strength gains owing to E2 activation of satellite cells that is supressed by P4 in the luteal phase. Following the evidence presented herein, this review serves to guide researchers in conceptualizing future studies where

research is lacking, and to test progressive interventions for female athletes to circumvent or gain advantage from the menstrual phase-specific responses.

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Author contributions

TO, JAS, and ACH, conceived the review design; TO drafted the manuscript; TO, JAS, and ACH edited, revised, and approved the final version of the manuscript.

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Figure captions

Fig. 1 Diagram defining the menstrual cycle phases showing typical serum 17-β-estradiol and progesterone concentrations

Fig. 2 Diagram showing the molecular structure of 17-β-estradiol (E2) and a schematic of how it protects plasma membrane integrity **(a)**, that is contrasted to the molecular structure of progesterone (P4) **(b)** which is not a structural antioxidant

Fig. 3 Diagram showing the receptor-activated signaling pathways of estrogen (E2) and progesterone (P4). The classical direct genomic effects **(a)** of E2 and P4 occur via cytosolic binding of respective nuclear receptor transcription factors, estrogen receptors (ER) and progesterone receptors (PR) in dimer complexes that translocate to the nucleus to regulate gene expression on binding their respective response elements (ERE or PRE, respectively) in the promoter of target genes or by binding certain DNA-bound transcription factors (TF) to transactivate or trans-repress target gene expression. In the same way, E2 regulates mitochondrial DNA expression. The indirect genomic and rapid non-genomic effects **(b)** occur via binding plasma membrane-bound receptors complexed with G-proteins, namely, ER and PR or alternate membrane receptors, G-protein coupled estrogen receptor (GPER) and progesterone receptor membrane complex 1 (PGRMC1), membrane progestin receptors (mPRs), respectively. On E2 and P4 binding, these membrane-bound receptors activate various signaling kinases that phosphorylate DNA-bound ER or PR or other TF to regulate target gene expression indirectly. In addition, the activated kinases bring about other rapid non-genomic cellular responses and membrane-bound ER signaling regulates endoplasmic/sarcoplasmic reticular calcium (Ca^{2+}) ion channel activity to increase cytosolic

Ca^{2+} content and related signaling. p38MAPK, p38 mitogen activated protein kinase; ERK1/2, extracellular signal-related kinases1/2, PI3K, phosphoinositide-3-kinase; Akt, RAC (Rho family)-alpha serine/threonine-protein kinase (also known as protein kinase B); AMPK, AMP-activated protein kinase

Fig. 4 Diagram showing E2 and P4 signaling to influence exercise carbohydrate metabolism. In skeletal muscle, E2 transactivates or trans-represses GLUT4 gene expression via ERα or ERβ, respectively, that bind specific DNA-bound transcription factors (1). GLUT4 translocation to the plasma membrane is promoted by E2 independent of insulin via membrane-bound ERα that directly stimulates phosphoinositide-3-kinase (PI3K), which then signals via protein kinase B (Akt) (2a) or Ras-related C3 botulinum toxin substrate 1 (Rac1) (2b) and via independent phosphorylation of AMPK (3). P4 antagonizes these E2 effects mainly by inhibition of ER expression and protein content. In hepatocytes, E2 via PI3k/Akt signaling phosphorylates forkhead box protein O1 (FOXO1) to cause its nuclear export and degradation and thereby trans-represses expression of gluconeogenic target genes (4). Conversely, in a glucose-deprived state typical of fasted exercise, P4 trans-activates expression of these gluconeogenic genes via binding membrane-bound PGRMC1 that activates adenylate cyclase and cyclic-AMP production that activates protein kinase A (PK-A) to phosphorylate and activate the transcription factor, cAMP-response element binding protein (CREB) (5). CEBPA, CCAAT/enhancer-binding protein alpha; G6Pc, glucose-6-phosphotase; IRS, insulin receptor substrate; NFκB, nuclear factor kappa-B; PDK1, phosphoinositide-dependent protein kinase-1; PEPCK, phosphenolpyruvate-carboxykinase; PIP3, PI-3,4,5-triphosphate; Rab, Rab-GTPase; SP-1, specific protein-1; TBC1D1/4, TBC1Domain family member1 or 4 (also known as AS160)

Fig. 5 Forest plot showing the mean difference (95% confidence interval, CI) of menstrual phase or estrogen (E2) supplement treatment on rate of plasma glucose uptake (Rd) **(a)** and rate of plasma glucose appearance (Ra) **(b)** during exercise when fasted or when fed; showing the heterogenicity indices, z-scores, p-values, and effect size (ES) for each outcome. Effect size scores are interpreted as: 0.0-0.2 is trivial; 0.2-0.6 is small; 0.6-1.2 is moderate; >1.2 is large effects, respectively

Fig. 6 Diagram showing E2 signaling to regulate fat availability away from adipose tissue and towards skeletal muscle lipid storage and oxidation. In skeletal muscle, depicted on the bottom right, E2 bound ERα activates gene expression of the nuclear hormone receptors, PPARα and PPARδ directly or indirectly via phosphorylation of AMPK. PPARs enhance expression of plasma membrane (fatty acid transport protein, FATP and fatty acid translocase, CD36), cytosolic (fatty acid binding protein cytosolic, FABPc) and mitochondrial (carnitine palmityltransferase 1, CPT1) LCFA transporters, beta oxidation enzymes (medium-chain acyl-CoA dehydrogenase, MCAD; trifunctional protein-alpha, TFPα; β-hydroxy-acyl-CoA dehydrogenase, β-HAD) and kinases (pyruvate dehydrogenase kinase 4, PDK4) that direct metabolism away from carbohydrate and in favor of fat oxidation. E2 also increases lipid availability in skeletal muscle first by increasing skeletal muscle localized lipoprotein lipase (LPL) to support plasma very low density lipoprotein triglyceride (VLDL-TG) hydrolysis for LCFA uptake, second by increasing the expression of the transcription factor, sterol regulatory element-binding protein 1c (SREBP1c) and downstream targets for skeletal muscle IMTG storage (mitochondrial glycerol-3-phosphate acyl transferase, mtGPAT; acetyl-CoA carboxylase, ACC; fatty acid synthase, FAS) and third E2

promotes a redistribution of LCFA away from adipose tissue and toward skeletal muscle, as depicted on the top left, by decreasing adipose-localized LPL activity, decreasing lipid storage by repressing SREBP1c expression and promoting adipose lipolysis by increasing β-adrenergic receptor (β-AR) sensitivity to epinephrine, decreasing α_2-AR sensitivity and directly or indirectly increasing perilipin activity. P4 antagonizes E2 effects possibly by repressing ER expression

Fig. 7 Forest plot showing the mean difference (95% confidence interval, CI) of menstrual phase on rate of plasma LCFA appearance (Ra), uptake (Rd), and oxidation (Rox) during exercise **(a)** and rate plasma glycerol appearance (Ra), and uptake (Rd) during exercise as an indication of whole body lipolytic flux **(b)**; showing the heterogenicity indices, z-scores, p-values, and effect size (ES) for each outcome. Effect size scores are interpreted as: 0.0-0.2 is trivial; 0.2-0.6 is small; 0.6-1.2 is moderate; >1.2 is large effects, respectively

Fig. 8 Forest plot showing the mean difference (95% confidence interval, CI) of menstrual phase on rate of plasma leucine oxidation (Rox) and plasma phenyalanine oxidation (Rox) at rest **(a)** and plasma leucine flux and plasma phenyalanine flux at rest **(b)** where rate of appearance (Ra) equals the rate of uptake (Rd); showing the heterogenicity indices, z-scores, p-values, and effect size (ES) for each outcome. Effect size scores are interpreted as: 0.0-0.2 is trivial; 0.2-0.6 is small; 0.6-1.2 is moderate; >1.2 is large effects, respectively

Table 1 Reference ranges* for serum 17-β-estradiol (E2) and progesterone (P4) concentrations that define the menstrual phases

Menstrual phase	Recommended timing	Serum E2 (pmol/L)		Serum P4 (nmol/L)		Evidence of ovulation
		Range of **Median** values	Range of **95%CI** values	Range of **Median** values	Range of **95%CI** values	
EF	days 1-7 from onset of menses	129-154	58-225	0.6-1.3	0.3-3.8	
MF	8 - 4 days before the day of ovulation	163-363	113-491	0.3-0.6	0.3-1.6	
LF	0 - 2 days before the day of ovulation (or LH surge)	651-939	337-1518	0.6-2.5	0.3-4.1	
ML	5 -10 days after ovulation	451-552	267-807	32.4-42.5	22.4-60.4	Require a minimum P4 >16 nmol/L

*Reference ranges are provided as the highest and lowest median values and highest and lowest 95% confidence interval (95%CI) values over the specified days according to findings by Stricker at al. (2006) from n = 20 eumenorrheic women (aged 20-36 years) who supplied daily blood samples for a complete menstrual cycle. *EF* early follicular, *LF* late follicular, *MF* mid-follicular, *ML* mid-luteal, *LH* luteinizing hormone