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Understanding the female athlete: molecular mechanisms underpinning

menstrual phase differences in exercise metabolism

Authors:

Tanja Oosthuyse^{1,2}*, Juliette A. Strauss³, Anthony C. Hackney⁴

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: <u>oosthuyse@polka.co.za</u>; <u>Tanja.Oosthuyse@wits.ac.za</u> 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

1 Abstract

2 Research should equitably reflect responses in both men and women. Including women in 3 research, however, necessitates an understanding of the ovarian hormones and menstrual 4 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 5 6 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 7 8 plasma membranes and quenching free-radicals and both E2 and progesterone (P4) promote 9 the expression of endogenous antioxidant enzymes attenuating exercise-induced muscle damage in certain menstrual phases. E2 and P4 bind nuclear hormone receptors and 10 membrane-bound receptors to regulate gene expression directly or indirectly through multiple 11 12 paths, which importantly includes cross-regulated expression of their own receptors. In addition, activation of membrane-bound receptors regulates kinases that feed into multiple 13 14 cellular pathways to bring about rapid cellular responses. Furthermore, receptor-type 15 dependent outcomes result in tissue-specific responses with variable inter-organ dependence. Careful analysis of these signaling pathways now explains the menstrual phase-specific 16 differences in hepatic glucose production, whole body plasma glucose uptake, tissue specific 17 lipid storage and fat oxidation, and protein catabolism during exercise, and post exercise 18 muscle satellite cell recruitment, activation, and proliferation. This review is expertly 19 20 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 21 actuality. Importantly, evidence-based relevance for training and performance in female 22 23 athletes and areas warranting further research exploration are highlighted.

- 25 Keywords: Premenopausal women, Eumenorrhea, Estrogen and progesterone signaling,
- 26 Exercise metabolism, Exercise-induced muscle damage
- 27

28 Abbreviations

29	ACC	acetyl-CoA carboxylase
30	Akt	protein kinase B
31	AMPK	5'AMP-activated protein kinase
32	AP-1	activator protein 1
33	BCOAD	branched-chain 2-oxo-acid dehydrogenase
34	cAMP	cyclic-AMP
35	CD36	fatty acid translocase
36	CEBPA	CCAAT/enhancer-binding protein alpha
37	СК	creatine kinase
38	CPT-1	carnitine palmitoyl transferase-1
39	CREB	cAMP-response element binding protein
40	DOMS	delayed onset muscle soreness
41	EIMD	exercise-induced muscle damage
42	E2	17-β-estradiol
43	EF	early follicular
44	ER	estrogen receptor
45	ERE	estrogen response elements
46	ERK	extracellular signal-related kinases
47	FABPc	fatty acid binding protein

48	FAS	fatty acid synthase
49	FATmax	exercise intensity at maximal fat oxidation
50	FATP	fatty acid transport protein
51	FOX01	forkhead box protein O1
52	FSH	follicle-stimulating hormone
53	FSR	fractional protein synthetic rate
54	G6Pc	glucose-6-phosphotase
55	GLUT4	glucose transporter 4
56	GnRH	gonadotropin-releasing hormone
57	GPER	G-protein estrogen receptor
58	β-HAD	β-hydroxyacyl-CoA dehydrogenase
59	IMTG	intramuscular triglyceride
60	IRS	insulin receptor substrate
61	LCFA	long chain fatty acid
62	LF	late follicular
63	LH	luteinizing hormone
64	LPL	lipoprotein lipase
65	MCAD	medium-chain fatty acyl-CoA dehydrogenase
66	MF	mid-follicular
67	ML	mid-luteal
68	mPR	membrane progestin receptors
69	mtGPAT	mitochondrial glycerol-3-phosphate acyl transferase
70	mtTFA	mitochondrial transcription factor A
71	Myo-D	myogenic differentiation factor D

72	ΝΓκΒ	nuclear factor kappa-B
73	NRF-1	nuclear respiratory factor-1
74	OC	oral contraceptive
75	OVX	ovariectomized
76	р38МАРК	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 TFP α trifunctional protein-alpha

101 Introduction

Since the early 1980s researchers have observed menstrual phase differences in the metabolic 102 103 response to exercise that can influence exercise capacity or performance (for example, 104 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 105 various aspects of carbohydrate, fat, and protein metabolism during exercise to isolate the 106 underlying modulations and the metabolic state that is required for these changes to be 107 108 notable (for reviews see, Oosthuyse and Bosch, 2010; Hackney 2021; Boisseau and Isacco 109 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 110 111 the menstrual phase-specific differences. Understanding the ovarian hormone metabolic signaling mechanisms will improve future research design striving to optimise training and 112 performance in female athletes. This review aims to describe the menstrual phase ovarian 113 hormonal effects on exercise metabolism while highlighting the molecular (signaling) 114 mechanisms. With that objective, this review: (i) defines the menstrual phases; (ii) presents 115 116 the molecular structure of estrogen as a mechanistic factor instrumental in regulating exercise-induced muscle damage (EIMD); (iii) introduces the receptor-activated signaling 117 pathways of estrogen and progesterone; (iv) reviews the effects of the menstrual cycle on 118 119 carbohydrate, fat and protein metabolism during exercise and the regulatory signaling mechanisms for each (including meta-analyses where appropriate to describe the magnitude 120 121 of effect on the oxidation or turnover of the various endogenous energy sources); and (v) 122 introduces the effect of the menstrual cycle on muscle regeneration and strength. 123 Additionally, practical relevance for female athletes and areas warranting further research exploration are highlighted. 124

125

126 **Defining the menstrual phases**

The ovarian status of women is multiplexed and requires methodological considerations 127 when conducting research in women (Elliott-Sale et al. 2021). The current review focuses on 128 premenopausal, eumenorrheic women who experience natural, ovulatory menstrual cycles. 129 The reproductive physiology of a eumenorrheic (normal, regular length, ovulatory) menstrual 130 cycle is comprehensively described by Mihm et al. (2011), where the predictable rise and fall 131 in the circulating ovarian hormones, estrogen, and progesterone, are explained in the context 132 133 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 134 hormone (GnRH), to co-ordinate follicle development and regression. This is a worthwhile 135 136 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 137 eumenorrheic cycle, which is partitioned into the following phases: early follicular (EF), mid-138 follicular (MF), late follicular (LF), ovulation, early luteal, mid-luteal (ML) and late luteal 139 phases (Fig. 1). It is noteworthy that while a large proportion of premenopausal women 140 141 experience menstrual cycles of ~28 days, the length of a eumenorrheic cycle can vary 142 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 143 144 longer cycle lengths follow a 3-wave follicular cycle, with respect to follicle development 145 that culminates in the selection of a dominant follicle. Thus, the longer menstrual cycle in 146 some eumenorrheic women is owing to a longer follicular phase whereas the luteal phase is 147 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 148 Jonge et al. (2019) has outlined what are deemed acceptable methods for predicting and 149 150 verifying menstrual phase and ovulation. Accordingly, menstrual phase comparative studies

are required to verify the menstrual cycle phase by reporting measured estrogen and 151 progesterone concentrations. To-this-end, only menstrual phase comparative studies that 152 153 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 20 154 eumenorrheic women and these median values can be used to guide researchers in defining 155 the acceptable concentration ranges when verifying menstrual phases in female participants 156 157 (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 158 159 is occasionally included in studies, presenting an ideal time to evaluate the effect of estrogen without progesterone. 160

161

insert Figure 1 and Table 1 near here

162

163 Estrogen modulates EIMD by virtue of its molecular structure

Estrogen and progesterone (P4) are synthesized primarily in the ovaries by canonical 164 165 steroidogenesis with cholesterol as the primary precursor, and to a lesser extent in other 166 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 167 168 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 169 170 androgens and finally the estrogens (see Häggström and Richfield 2014). The three main endogenous estrogens include: estrone (E1), 17-\beta-estradiol (E2), and estriol (E3), with a 171 fourth estrogen, estetrol (E4) only present during pregnancy, where the assigned numeral 172 defines the number of hydroxyl groups. E2 is the most potent and prominent form that is 173 174 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).

insert Figure 2 near here

184

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

damage and membrane instability (Tiidus 2003; Enns and Tiidus 2010). For this reason, the 199 change in serum CK activity following exercise is a marker of sarcolemma stability. In a 200 201 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 202 and this protection increased markedly with the duration of estrogen therapy (reviewed by 203 Tiidus 2003; Enns and Tiidus 2010). Similarly, estrogen treatment reduced the influx of 204 205 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 206 207 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 208 peptides which explains the decrease in neutrophil infiltration and overall reduced muscle 209 210 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 211 serum CK activity and post exercise CK response have been reported with lower resting CK 212 levels in women and reduced post exercise CK response (Sewright et al. 2008; Luk et al. 213 2021; Kumagai et al. 2022) or quicker return to resting levels in women than men (Oosthuyse 214 and Bosch 2017). Furthermore, given that population variability in the genotypes of alleles 215 216 within genes can translate into some differences in the functionality of the expressed protein, 217 it is interesting that men having a TT genotype polymorphism in the gene for the E2 218 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 219 evidence showed women to have a muted intramuscular cytokine response to muscle 220 221 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). 222 Interestingly, while the serum CK and delayed onset muscle soreness (DOMS) recovery 223

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).

228

229 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 230 231 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 232 E2 concentrations in eumenorrheic women who participated in either their EF, LF or ML 233 234 phase (Oosthuyse and Bosch 2017). Likewise, serum CK activity was consistently lower 24 h 235 and 72 h after 60-90 min of level running at 70% VO₂peak in the ML compared with MF phase (Williams et al. 2015; Hackney et al. 2019); with a negative correlation between the 236 237 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 238 phase (Hackney et al. 2019). A recent meta-analysis attempted to quantify the effect of the 239 menstrual phase on markers of EIMD by including all prior studies in females and found no 240 241 clear menstrual phase effect on the serum CK response (Romero-Parra et al. 2021). However, 242 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 243 suggests the outcome of that meta-analysis should be graded as low certainty and interpreted 244 245 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 246 247 independent of menstrual phase. Furthermore, when an unaccustomed mechanical stress is extremely high, such as during maximal force eccentric exercise, the severity of structural 248

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.

256

The cellular protective effect of E2 persists even in the presence of the estrogen receptor 257 inhibitor, tamoxifen (Paroo et al. 2002), supporting the functional role of the molecular 258 259 structure of E2 to be instrumental in conferring these benefits independent of certain typical 260 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. 261 262 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. 263 2008). And therefore, the antioxidant role of E2 appears to be a sum of its role as both a 264 structural antioxidant and genomic activator. Agreeably, a recent study quantified the 265 presence of reactive oxygen metabolites and tested the antioxidant potential in blood samples 266 267 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 268 metabolites in the EF, but not ML phase after exercise, together with a tendency for a greater 269 270 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. 271

P4, unlike E2, is not a structural antioxidant and is in fact susceptible to reduction at the 273 ketone groups or carbon double bond (positioned between C4 and C5; from where the 274 abbreviation, P4 derives its numeral) (Fig. 2b). P4, however, has been shown to reduce free 275 radical damage by increasing the expression and activity of the antioxidant defence enzymes, 276 SOD, glutathione peroxidase, and glutathione reductase via P4 receptor signaling 277 (Hernández-Rabaza et al. 2019). For this reason, the synergistic effect of E2 and P4 in the 278 279 ML phase to reduce oxidative stress supports evidence for the ML phase to be better than the EF phase for exercise that risks EIMD. 280

281

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).

288

289 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.

297 Nuclear hormone receptors

298 Both E2 and P4 influence metabolism by binding estrogen and progesterone nuclear hormone receptors that occur as E2 receptor-alpha (ER α) and E2 receptor-beta (ER β) or P4 receptor-A 299 (PR-A) and P4 receptor-B (PR-B) isoforms, respectively. Receptor isoform specificity is 300 relevant because the various receptor isoforms are linked to differing metabolic outcomes 301 302 (Foryst-Ludwig and Kintscher, 2010; Mauvais-Jarvis et al. 2013; Gregorio et al. 2021). E2 303 and P4 bind to the respective isoforms with equal affinity and the net outcome is, therefore, 304 dependent on the tissue-specific isoform abundance (Yaşar et al. 2016). E2 and P4 diffuse 305 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 306 typically as dimer complexes to act as ligand-activated transcription factors and bind estrogen 307 response elements (ERE) or progesterone response elements (PRE), respectively, in the 308 309 promoter regions of target genes to activate or repress gene transcription (Yaşar et al. 2016; 310 Puglisi et al. 2019). E2 and P4 bound to their cognate nuclear hormone receptors can also 311 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 312 313 and Kintscher, 2010; Yaşar et al. 2016; Puglisi et al. 2019; Gonzalez, 2020).

314

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

320	transcription factor A (mtTFA) (Yaşar et al. 2016; Ventura-Clapier et al. 2019). In this way,
321	E2 promotes mitochondrial biogenesis and capacity for oxidative phosphorylation. Both ER α
322	and $\text{ER}\beta$ isoforms have been identified in mitochondria as well as membrane-bound E2
323	receptors (Ventura-Clapier et al. 2019).

325 Membrane-bound receptors

E2 and P4 induce rapid signaling in seconds or minutes by binding membrane-bound 326 327 receptors coupled to G proteins that activate various kinases, namely: phosphoinositide-3kinase (PI3K)/Akt, extracellular signal-related kinases (ERK), p38 mitogen activated protein 328 kinase (p38MAPK), and others, and thereby E2 and P4 act in a non-genomic manner to 329 phosphorylate and regulate the activity of other cellular proteins (Puglisi et al. 2019; 330 Gonzalez, 2020). The activated kinases can also phosphorylate nuclear bound E2 or P4 331 332 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 333 binding (Yaşar et al. 2016; Puglisi et al. 2019). Similarly, the activated kinases can 334 phosphorylate various other nuclear bound transcription factors to indirectly regulate gene 335 expression of other target genes lacking ERE or PRE and without any interaction of E2 and 336 P4 nuclear hormone receptors (Yaşar et al. 2016; Puglisi et al. 2019). In this way, E2 and P4 337 338 can impose indirect genomic regulation when binding their membrane-bound receptors. Furthermore, E2 regulates calcium ion channels and sarcoplasmic/endoplasmic reticular 339 calcium transporters to modulate cytosolic and mitochondrial calcium content and associated 340 signaling cascades (Mahmoodzadeh and Dworatzek, 2019; Puglisi et al. 2019). E2 is also 341 functional in the phosphorylation of 5'AMP-activated protein kinase (AMPK) and rapidly 342 increases its activity within less than 10 min of administering E2 in vitro or in situ (D'Eon et 343

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.

350

It is proposed that the membrane-bound receptors can include: (1) the classical E2 and P4 351 352 nuclear hormone receptors that are in this instance membrane-bound and (2) further receptor 353 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 354 receptors (mPR) (Garg et al. 2017; Gonzalez et al. 2020; Medina-Laver et al. 2021). Recent 355 evidence in adipocytes suggests that of the E2 classical nuclear hormone receptor isoforms, 356 only ER α translocate to the plasma membrane in the presence of E2, whereas ER β remain 357 nuclear bound (Campello et al. 2017; Gregorio et al. 2021). For this reason, it is likely that E2 358 membrane-receptor signaling, and rapid non-genomic effects occur mostly via specifically 359 360 E2-ER α or E2-GPER interactions and not E2-ER β ; albeit possibly tissue specific. Studies in 361 mice report expression of $ER\alpha > GPER > ER\beta$ in both soleus (type 1 fibers) and extensor digitorum longus (type 2 fibers) muscles (Baltgalvis et al. 2010) and expression of ER α > 362 ER β in not only skeletal muscle but also liver and adipose (Ribas et al. 2010). 363

364

insert Figure 3 near here

365

366 P4 represses ER expression

Evidence exists for E2 and P4 cross-interactions that are often antagonistic (Campbell and 367 Febbraio, 2001, 2002) with the net outcome in the luteal phase dependent on the relative 368 369 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 370 and Pike 2009; Ekenros et al. 2017). In an in vitro study in neurons, E2 promoted ERa and 371 ERβ expression and P4 downregulated this effect rapidly within 15 min and the level of 372 repression increased with greater incubation time (Jayaraman and Pike 2009). P4 repression 373 was maximal at typical ML concentrations of 30 nmol/L (Jayaraman and Pike 2009). 374 375 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 376 and protein content was found to be lowest in the ML phase (Ekenros et al. 2017). The order 377 378 of skeletal muscle ER α mRNA content occurred as: EF > ovulation > ML that coincided with 379 typical serum P4 concentrations that were lowest in EF, modestly higher at ovulation, and substantially higher in ML. Accordingly, ERa protein content was greater in the EF phase 380 than ML phase. In that study, the modest serum E2 concentrations reported during the 381 ovulation sampling period (200 pmol/L) might suggest that sampling may have occurred in 382 383 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 384 385 concentrations. Moreover, PR protein content was found to be greater in the ML than both at 386 ovulation and EF phase suggesting that P4 promotes PR abundance, which conversely does 387 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. 388 389 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 390 interpreting outcomes (D'Eon et al. 2002; Horton et al. 2002). 391

393 Mechanisms behind menstrual cycle effects on carbohydrate metabolism

394 Overview of exercise-stimulated plasma glucose uptake

Our understanding of insulin signaling, and exercise-stimulated plasma glucose uptake has 395 advanced. The basic framework of the insulin signaling pathway includes insulin binding to 396 membrane-bound insulin receptor tyrosine kinase that phosphorylates insulin receptor 397 398 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) 399 and initiates two signaling pathways. Firstly, the PI3K pathway phosphorylates and activates 400 401 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 402 AS160), and thereby relieves inhibition on Rab-GTPase that then facilitates translocation of 403 404 GLUT4 storage vesicles to the plasma membrane (Sakamoto and Holman 2008). Secondly, 405 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 406 translocation and therefore plasma glucose uptake (Yue et al. 2020). Exercise-stimulated 407 plasma glucose uptake acts independently of insulin and the current viewpoint is that it is 408 partly reliant on AMPK to directly phosphorylate TBC1D1/4 (de Wendt et al. 2021); 409 although evidence suggests that while AMPK is essential for increasing glucose uptake after 410 exercise, it is not essential during exercise (Kjøbsted et al. 2019; McConell, 2020). Instead, 411 412 exercise-stimulated glucose uptake is promoted by mechanical stress or stretch that activates integrin receptors and focal adhesions, or dystrophin-glycoprotein complexes, which then 413 promote Rac1 activity and GLUT4 translocation (Sylow et al. 2017; Flores-Opazo et al. 414 2020) (Fig. 4). However, it is also possible that the AMPK and Rac1 pathways driving 415

416	exercise-stimulated glucose uptake are inter-related (Yue et al. 2020; de Wendt et al. 2021).
417	A recent viewpoint suggests that the increase in glucose uptake with exercise is not only
418	dependent on GLUT4 translocation but also possibly an increase in GLUT4 activity (Richter,
419	2021). Furthermore, during exercise it can be assumed that all of the plasma glucose taken up
420	is shuttled into glycolysis and oxidation (Sylow et al. 2017) and therefore the measured rate
421	of plasma glucose uptake is often used as a surrogate for the rate of plasma glucose utilization
422	during exercise.
423	***insert Figure 4 near here***
424	
425	E2 and P4 influence of plasma glucose uptake
426	E2 and P4 signaling feed into insulin signaling pathways (Wada et al. 2010; Mauvais-Jarvis
427	et al. 2013) and in addition E2 influences GLUT4 expression (reviewed by Gregorio et al.
428	2021) to affect glucose uptake (Fig. 4), as outlined in the sections that follow.
429	
430	E2 regulation of GLUT4 expression
431	While there is no ERE in the promoter of the gene (solute carrier family 2 member 4, <i>Slc2a4</i>)
432	encoding GLUT4, E2-ER exerts genomic effects by binding various Slc2a4 transcription
433	factors (Gregorio et al. 2021). E2 influence is dependent on the ER isoform where E2-ER β
434	trans-represses and E2-ER α transactivates GLUT4 expression (Campello et al. 2012;
435	Barreto-Andrade et al. 2018). For example, E2-ER α binds the inflammation-stimulated
436	transcription factor, nuclear factor kappa-B (NF κ B), and thereby blocks its repression of
437	GLUT4 expression (Campello et al. 2012); E2-ER α binds specific protein 1 (SP-1) on the
438	promoter of <i>Slc2a4</i> causing transactivation of GLUT4 expression (Barreto-Andrade et al.

439 2018); E2-ER α binds CCAAT/enhancer-binding protein alpha (CEBPA) to transactivate 440 GLUT4 expression and also directly increases the expression and content of CEBPA and 441 promotes its translocation to the nucleus (Fatima et al. 2019). The mechanism explaining 442 ER β trans-repression of GLUT4 expression is not clear but may be via inhibition of SP-1 443 (Gregorio et al. 2021).

444

P4 alone did not change adipocyte GLUT4 expression in vitro (Wada et al. 2010) but did 445 reduce GLUT4 protein content in adipose tissue and skeletal muscle of OVX rodents 446 compared with sham-operated rodents (Campbell and Febbraio, 2002). In fact, GLUT4 447 content was suppressed even when P4 was administered with E2 and P4-inhibition was only 448 449 overcome with a supra-physiological E2 dose (Campbell and Febbraio, 2002). Thus, P4 450 effect on GLUT4 expression or protein content may be indirect possibly in part due to P4-451 repression of ERa expression. Accordingly, skeletal muscle GLUT4 mRNA content was greater in women in their follicular phase compared with men and was increased 2-fold in 452 men after 8 days of oral E2 supplementation that mimicked LF phase serum E2 453 concentrations (946 pmol/L) (Fu et al. 2009). Furthermore, a comparison between menstrual 454 phases revealed a trend for 2.2-fold greater skeletal muscle GLUT4 mRNA content during 455 456 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 457 late luteal phase and possibly represented a P4 dominant effect. 458

459

460 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;

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

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

474

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

486 likely presenting the most favourable time for this E2-dominant effect to be realized and487 certainly warrants investigation in eumenorrheic athletes.

488

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.

493

494 Whole-body plasma glucose uptake during exercise

495 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. 496 2002) or fasted (Campbell et al. 2001; Zderic et al. 2001; Horton et al. 2002; Devries et al. 497 2006) eumenorrheic women, or after purposeful controlled manipulation of ovarian hormone 498 concentrations in non-OC users (D'Eon et al. 2002), or in amenorrheic women (Ruby et al. 499 500 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 501 SD) rate of plasma glucose uptake measured in µmol/kg·min in the EF phase/placebo trial is 502 compared with the ML phase/hormone supplemented trial in each study to derive a mean 503 504 difference and effect size as the standardised mean difference using Review Manager (RevMan version 5.4.1) (Fig. 5a). 505

506

507 While most studies included in the meta-analysis did in fact apply a within-participant
508 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 509 presented reflect independent between-group and not dependent within-group outcomes and 510 511 the meta-analysis outcome likely underestimates the true effect, adopting a conservative approach. In all studies, stable isotopic-tracer methodology was applied by constant 512 intravenous infusion of [6,6-²H]glucose to attain steady-state enrichment before plasma 513 glucose rate of appearance (Ra) and rate of disappearance (Rd) could be derived from 514 515 validated equations based on the principles of tracer dilution (Kim et al. 2016), where glucose 516 Rd represents the rate of whole-body plasma glucose uptake. The meta-analysis outcome 517 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 518 the EF phase, with a moderate effect during fasted exercise and a small non-significant effect 519 when exercising fed (Fig. 5a). Thus, demonstrating that ovarian hormone effects are more 520 notable during periods of metabolic stress or a high demand for endogenous substrates. 521 Second, while we may expect conditions of short-term E2 treatment alone in men or 522 amenorrheic women to increase the rate of plasma glucose uptake during exercise, instead 523 studies also report a significant decrease with E2 treatment compared with placebo (Fig. 5a). 524 This seemingly discrepant finding may be a result of men and amenorrheic women, who 525 experience chronic low E2 concentrations, also having low ERa content in skeletal muscle 526 and greater relative ER^β content as is typical in E2 deficient conditions, such as menopause 527 (Foryst-Luwig and Kintscher, 2010; Park et al. 2017). However, in the study by D'Eon et al. 528 (2002), where ovarian hormones in eumenorrheic women were suppressed with a GnRH 529 antagonist followed by treatment with E2 alone or combined E2 and P4, the rate of plasma 530 glucose uptake during exercise also tended to be reduced with E2 treatment (or E2 plus P4) 531 compared with ovarian suppression. Thus, suggesting that despite prior evidence for tissue-532 specific effects in skeletal muscle for E2 to increase exercise-stimulated glucose uptake, on a 533

whole-body level E2 reduces the rate of plasma glucose uptake during exercise. The current 534 535 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 536 on the rate of plasma glucose uptake during exercise lasting 50-120 min at 50-70% VO₂max 537 (Fig. 5a), that equates to -2.7 g/h of glucose taken up for 60 kg women. Such a magnitude of 538 effect may appear to have little physiological relevance over the duration of exercise that has 539 been tested, but it remains to be determined whether longer duration exercise produces larger 540 and more meaningful menstrual phase effects. 541

542

insert Figure 5 near here

543

Moreover, oral contraceptive therapy for 4 months has been found to reduce the rate of 544 plasma glucose uptake during exercise by up to 20% (-8.44 µmol/kg·min) even when 545 exercising fed (3 h postprandial) compared with before-OC use in eumenorrheic women (Suh 546 et al. 2003). It may be that OC potency is greater than the naturally cycling endogenous 547 hormones resulting in a larger effect that becomes notable even with less metabolic stress. 548 Such a decrease in the rate of plasma glucose uptake with OC use versus non-OC use equates 549 to -5.5 g/h of glucose taken up for 60 kg women, an amount that represents 5% of total 550 carbohydrate oxidation (reported as 1.75 g/min or 105 g/h) during exercise at 65% VO₂max 551 552 (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 553 receptor binding and downstream signaling responses in hepatocytes (Magkos et al. 2022). 554 555

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 558 conditional outcomes on the rate of plasma glucose appearance during exercise mimics the 559 560 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 561 derived from hepatic glycogenolysis and gluconeogenesis. Considering evidence for E2 to 562 increase skeletal muscle-specific glucose Rd capacity during exercise, and yet whole-body 563 564 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 565 566 by plasma glucose Ra and the influence of E2 (and P4) on hepatic glucose production.

567

568 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 569 gluconeogenesis on provision of isotopic-glucogenic precursors at rest (Matute and Kalkhoff, 570 571 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 572 ERα inhibitor found that E2 suppresses gluconeogenesis and thus hepatic glucose production 573 574 (Yan et al. 2019). E2 acts by binding ER α at the plasma membrane to induce PI3K and Akt 575 phosphorylation, where Akt, in turn, phosphorylates forkhead box protein O1 (FOXO1) 576 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 577 phosphenolpyruvate-carboxykinase (PEPCK) (Yan et al. 2019). In this way, E2 like insulin, 578 represses the expression of these gluconeogenic enzymes (Fig. 4). 579

Conversely, the P4 effect on gluconeogenesis is variable depending on carbohydrate 581 availability (Lee et al. 2020). With carbohydrate provision, P4 enhances insulin secretion to 582 suppress gluconeogenesis in healthy insulin-sensitive states, as seen in male mice with P4 583 treatment (Lee et al. 2020). Alternatively, in a glucose-depleted condition, as may occur 584 during fasted exercise, P4 in hepatocytes increases glucose production by increasing the 585 expression of the key gluconeogenic enzymes (G6Pc and PEPCK). An in vitro model using 586 587 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, 588 589 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, 590 considering plasma glucose Ra during fasted exercise is reduced in the ML phase, we can 591 assume that in eumenorrheic women, E2 suppression of hepatic glucose production 592 predominates. 593

594

595 Overriding hepatic glucose production annuls the decrease in plasma glucose uptake 596 during exercise in the ML phase

597 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% VO₂max during the EF and ML 598 599 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 600 the ingested supplement with hepatic glucose production contributing minimally. With a 601 602 surplus plasma glucose supply, the rate of plasma glucose uptake increased similarly in both 603 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. 604

Furthermore, a recent study overcame the limitation of E2 or P4 influence on 606 607 gluconeogenesis and hepatic glucose production by implementing an intravenous 10 mM hyperglycemic clamp during 90 min of exercise at 60% VO₂max in the follicular phase and 608 luteal phase and the rate of glucose uptake was inferred from the rate of glucose infusion 609 610 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 611 represents early luteal or late luteal phase in some subjects as serum E2 concentration 612 increasing from a mean ~100 pmol/L in follicular phase to ~200 pmol/L in luteal phase and 613 serum P4 concentration attaining only a mean of 14 nmol/L in luteal phase with P4 increasing 614 above the 16 nmol/L threshold to confirm an ovulatory cycle in only 2 of the 7 participants. 615 Notwithstanding, this study also found that the rate of plasma glucose uptake during exercise 616 617 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 618 619 for a 60 kg women) (Hulton et al. 2021). These findings remain to be confirmed by further 620 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). 621

622

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 80 629 min of moderate-hard exercise in a crossover design matched for the menstrual phase, 630 metabolism shifted in favour of greater carbohydrate oxidation that coincided with a small 631 increase in distance covered and total work done during the final 20 min time trial 632 (Oosthuyse et al. 2021). Thus, the restriction of E2 (and P4) on gluconeogenesis during the 633 ML and possibly LF phases can have negative implications for exercise performance under 634 635 certain conditions of prolonged exercise requiring high intensity efforts without adequate carbohydrate supplementation. For this reason, the ability to store muscle and liver glycogen 636 637 is also important.

638

639 Influence of the ovarian hormones on glycogen storage and use

Studies have found a greater ability to replete muscle glycogen stores after glycogen 640 depleting exercise in the ML phase compared with MF phase (Nicklas et al. 1989; Hackney 641 1990) and when following a normal mixed diet including ~5 g/kg carbohydrate (MF: 575 642 mmol/kg d.w. vs. ML: 728 mmol/kg d.w.) (McLay et al. 2007). Although this menstrual 643 644 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 645 (1.2 g/kg per hour) during the immediate 5 h recovery period after glycogen depleting 646 647 exercise, a similar muscle glycogen repletion is achieved in EF, LF, and ML phases (Matsuda 648 et al. 2022b). E2 increases glycogen synthase activity (Beckett et al. 2002) possibly by acting 649 via membrane-bound ERa to stimulate PI3K/Akt signaling to phosphorylate glycogen synthase kinase- 3α thereby releasing its inhibition on glycogen synthase activity (Yan et al. 650 2019) and by increasing glycogen synthase expression as seen in men after 8 days of oral E2 651 supplementation (Fu et al. 2009). However, it may be less relevant when following a high 652

653 carbohydrate-rich diet and during acute recovery when ingesting a high-dose carbohydrate654 supplement that results in an already maximal insulin-stimulated signaling response.

655

Muscle glycogen use during moderate-intensity endurance exercise (60-70% VO₂max) based 656 on analysis of muscle biopsies has been reported to be 24% and 25% less in the ML versus 657 EF phase in two independent studies, respectively, coincident to greater fat oxidation 658 (Hackney 1999; Devries et al. 2006) where glycogen use was inversely correlated with E2 659 660 concentration in the ML phase (Hackney 1999). Likewise, the rate of glycogen use at 60% VO₂max, estimated from the difference between the rate of total carbohydrate oxidation 661 and tracer measured plasma glucose Rd, was 24% and 28% less in eumenorrheic women on 662 663 controlled E2 supplements mimicking the LF phase also coincident to greater fat oxidation 664 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 665 666 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 667 to greater fat oxidation during moderate-intensity endurance exercise and can be countered by 668 P4 and hence is dependent on the relative increase in E2 and P4 (Hackney et al. 2022). 669 Conversely, high-intensity exercise necessitates carbohydrate-sourced energy production, and 670 671 consequently, E2 suppression of hepatic gluconeogenesis might explain recent findings for greater muscle glycogen use, measured by ¹³C-magnetic resonance spectroscopy, during 672 overnight-fasted high-intensity intermittent exercise to exhaustion in the LF than EF phase 673 674 (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 675 absolute starting or ending muscle glycogen concentration (Matsuda et al. 2022a). 676

678 Mechanisms behind menstrual cycle effects on fat metabolism

679 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).

687

Subsequently, a collection of studies largely from three different laboratories have uncovered 688 the molecular signaling pathway whereby E2 promotes fat oxidation (Fig. 6). The cumulative 689 findings from these studies that employed either an OVX-rodent model, in vitro cell 690 incubation, men on E2 supplements or sex-differences have been reviewed in detail 691 692 elsewhere (Tarnopolsky 2008; Oosthuyse and Bosch, 2012). Briefly, in skeletal muscle, E2 bound ER α activates gene transcription of the nuclear hormone receptors, peroxisome 693 proliferation activator receptor-alpha (PPAR α) and PPAR δ either directly by binding ERE in 694 the gene promoter regions or indirectly via membrane bound $ER\alpha$ to induce phosphorylation 695 of AMPK (Campbell et al. 2003; D'Eon et al. 2005, 2008; Fu et al. 2009; Salehzadeh et al. 696 2011). PPAR α and PPAR δ activate the expression of downstream targets (some of which 697 may also be under direct genomic regulation of E2) to increase the protein content of plasma 698 membrane (fatty acid transport protein (FATP), fatty acid translocase (CD36)), cytosolic 699

700 (fatty acid binding protein (FABPc)) or mitochondrial (CPT-1) LCFA transporters and 701 mitochondrial beta oxidation enzymes (medium-chain fatty acyl-CoA dehydrogenase (MCAD); β -HAD; beta oxidation enzyme complex, trifunctional protein-alpha (TFP α)) and 702 the glycolytic down-regulator, pyruvate dehydrogenase kinase-4 (PDK4) to direct substrate 703 704 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 705 the Rab-GTPase activating proteins, TBC1D1/4 that regulate GLUT4 translocation are also 706 involved in regulating LCFA transporter content and trafficking to the plasma membrane and 707 thus LCFA oxidation (Benninghoff et al. 2020; Mikłosz et al. 2021). TBC1D1/4 maintains 708 the Rab GTPase proteins in an inactive GDP-bound state and this inhibition is released upon 709 phosphorylation of TBC1D1/4 by its upstream effectors (Akt and AMPK), which then allows 710 711 Rab-GTP binding and hydrolysis to facilitate transporter translocation. In this way, investigations are needed to establish whether E2 via membrane bound ER α phosphorylation 712 of PI3K/Akt and AMPK may possibly also contribute to LCFA transporter translocation and 713

714 increased LCFA uptake in skeletal muscle.

715

716 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 717 (SREBP1c), which activates gene expression of downstream targets of skeletal muscle 718 719 intramuscular triglyceride (IMTG) storage, namely, mitochondrial glycerol-3-phosphate acyl transferase (mtGPAT) for re-esterification and acetyl-CoA carboxylase (ACC) and fatty acid 720 synthase (FAS) for lipogenesis (Fu et al. 2009). Secondly, E2 via increased PPARS increases 721 expression and content of skeletal muscle localized lipoprotein lipase (LPL) to support 722 hydrolysis of plasma triglyceride for skeletal muscle LCFA uptake (D'Eon et al. 2005). 723 Thirdly, E2 promotes a redistribution of LCFA away from adipose tissue and toward skeletal 724

muscle by reducing adipose tissue localized LPL activity to reduce triglyceride uptake in 725 adipose (Ellis et al. 1994; Homma et al. 2000) and by reducing adipose-specific lipid storage 726 via tissue-specific inhibition of SREBP1c expression in adipose and liver (D'Eon et al. 2005) 727 and by promoting adipose triglyceride lipolysis by enhancing beta-adrenergic sensitivity to 728 epinephrine (Benoit et al. 1982), reducing antilipolytic alpha₂-adrenergic responsiveness 729 (Schmidt et al. 2014) and increasing perilipin protein content (albeit without identifying 730 subtype specificity) (D'Eon et al. 2005). The role of different perilipin protein isoforms in the 731 regulation of triglyceride storage and lipolysis in various tissues and sex-differences in 732 733 perilipin content have been reviewed (MacPherson and Peters 2015). The actions of perilipins are important for preventing the accumulation of lipid oxidation intermediates and 734 accordingly, perilipin actions differ from the basal state to the hormone-stimulated lipolytic 735 state (MacPherson and Peters 2015). 736

737

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).

743

insert Figure 6 near here

744

745 Menstrual cycle effect on whole-body fat oxidation

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

Reviews see, Devries, 2016; Boisseau and Isacco 2022; Cano et al. 2022). Surprisingly, 748 however, menstrual phase differences are less consistent, with only some reporting a greater 749 750 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 et al. 2001; Willett et al. 2021). However, E2 promotion of fat oxidation during exercise is 752 confirmed with men on E2 supplements (Hamadeh et al. 2005) and, as already mentioned, in 753 754 premenopausal women on controlled E2 treatment versus ovarian suppression or combined E2 and supraphysiological P4 treatment (D'Eon et al. 2002). In fact, the change in the rate of 755 756 fat oxidation during exercise from EF to ML phase has been found to correlate positively with the change in the E2/P4 ratio (Hackney et al. 2022). Moreover, in one study, a greater 757 rate of fat and lower carbohydrate oxidation in the ML versus MF phase was significant at 758 759 low (35% VO₂max) and moderate (60% VO₂max) exercise intensities but not significant at a higher intensity (75% VO₂max) (Hackney et al. 1994) possibly where a higher rate of 760 carbohydrate utilization becomes obligatory. Therefore, it seems that a notable shift in whole-761 body substrate utilisation with the menstrual phase is only evident: (i) during fasted exercise; 762 or (ii) mainly when exercise duration places a sufficiently high demand on endogenous 763 energy stores; and (iii) possibly only at or below the exercise intensity marking the metabolic 764 threshold after which carbohydrate utilization predominates; and (iv) as such is dependent on 765 a high relative concentration of E2 to P4 in the ML phase. 766

767

For example, the exercise intensity eliciting maximal fat oxidation (FATmax) and the peak
fat oxidation rate is not different between MF, LF and ML phases when measured in a
standard 3-min step incremental test to exhaustion (Frandsen et al. 2020). This is not
surprising because the 3-min steady-state steps are too short to pressurize energy metabolism
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.

779

780 Fat oxidation source: IMTG or plasma LCFA-derived

Sex-differences in fat oxidation may be partly owing to greater IMTG content in women 781 (because of a greater number of lipid droplets and not greater lipid droplet size) and greater 782 783 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 784 compared with men matched for training status (Cardinale et al. 2018; Montero et al. 2018), 785 that are E2-attributable perturbations according to signaling mechanisms already discussed. 786 Albeit early variations in methods of IMTG quantification (i.e., biochemical extraction, 787 788 immunofluorescence microscopy or proton magnetic resonance spectroscopy) have resulted in an unclear consensus for sex-differences in IMTG use during exercise (Steffensen et al. 789 2002; Roepstorff et al. 2002, 2006; White et al. 2003; Devries et al. 2007). Furthermore, 790 791 whether the cyclical fall and rise in E2 with the respective menstrual phases notably alters 792 IMTG content, sub-cellular location and use during exercise in eumenorrheic women has not 793 been evaluated and certainly warrants investigation. Improvements in histological staining 794 techniques has advanced our understanding of IMTG myofibrillar-localization and 795 association with perilipin proteins, IMTG utilization during exercise and resynthesis in

recovery (Jevons et al. 2020; Strauss et al. 2020) but are yet to be applied in menstrual phasecomparative studies.

798

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

816

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

change between EF and ML phases in glycerol kinetics (Casazza et al 2004) and no change in 820 the rate of plasma LCFA appearance, uptake, or oxidation (Jacobs et al. 2005) at either 821 822 45% VO₂max or 65% VO₂max. However, the small sample size (n = 5) and high variability in especially plasma LCFA kinetics and oxidation in the ML phase encourages further follow up 823 studies. Furthermore, in this former study the women participated fed (3 h postprandial) and 824 exercise lasted only 60 min, which might also partly explain the lack of differences. The 825 826 second study included a larger sample of women (n = 11) who were overnight-fasted and exercised for 90 min at 50% VO₂max, but some menstrual phase timing errors resulted in a 827 828 few missing data points and data being evaluated using a less sensitive unpaired groups design (Horton et al. 2006). Nonetheless, this latter study also reports no differences between 829 EF, MF and ML phases in the rate of plasma glycerol or LCFA appearance and uptake during 830 exercise but was without measurement of plasma LCFA oxidation. The findings from these 831 two studies have been combined in a summative meta-analysis that may encourage further 832 studies to investigate menstrual cycle effects on particularly plasma LCFA oxidation and flux 833 during exercise (Fig. 7a). This meta-analysis, however, does present a clear outcome for no 834 effect on plasma glycerol Ra and Rd, reflecting an unchanged whole-body lipolytic rate 835 during exercise between EF and ML phases in these studies (Fig. 7b). In addition, E2 836 treatment in amenorrheic women for 6 days (Ruby et al. 1997) or men for 8 days (Carter et al. 837 2001) failed to alter plasma glycerol kinetics during exercise further supporting the likelihood 838 of no notable ovarian hormone effect on whole-body lipolysis between menstrual phases or 839 with short-term E2 treatment. Of note, the above studies also all failed to find a difference in 840 total whole-body fat oxidation during exercise between menstrual phases or ovarian 841 conditions. It could be that the serum E2 concentration recorded in the ML phase in these 842 studies (E2: 311 pmol/L, P4: 34.7 nmol/L and E2: 393 pmol/L, P4 36.6 nmol/L, respectively) 843 was not high enough for ovarian or menstrual phase effects on fat metabolism to become 844

apparent; compared with studies that have reported a shift in substrate utilisation towards 845 greater fat oxidation in the ML phase (for example, E2: 519 pmol/L, P4 47.9 nmol/L (Willet 846 et al. 2021) where the change in fat oxidation from EF phase correlated with the change in the 847 E/P ratio (Hackney 2022). Others have suggested that the latter studies where E2 treatment 848 was administered (Ruby et al. 1997; Carter et al. 2001) were likely underpowered for the 849 given variability considering that a clear shift towards greater whole-body fat oxidation 850 851 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 852 853 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 854 ML compared with EF phase is evident. 855

insert Figure 7 near here

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Conversely, OC use for 4 months increased plasma cortisol concentration and whole-body 858 lipolysis during exercise, as indicated by stable-tracer measured plasma glycerol Ra that 859 860 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 861 FFA re-esterification and less plasma FFA being oxidized during exercise, measured by 862 plasma [1-¹³C]palmitate tracer kinetics, after 4 months of OC use compared with before OC 863 use and hence indicates a futile cycle of substrate mobilization (Jacobs et al. 2005). Yet 864 again, this demonstrates that OC use influences exercise metabolism differentially compared 865 866 with naturally cycling endogenous ovarian hormones experienced by eumenorrheic women, where differences also extend to other physiological parameters, such as basal inflammatory 867

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

870

Notably, the technical difficulties of administering LCFA tracers by intravenous infusion 871 could be a deterring factor to explain the shortage of such menstrual phase comparative 872 studies. The hydrophobic nature of LCFA tracers necessitates constitution with 5% human 873 serum albumin as a carrier protein that could pose ethical risk limitations. Furthermore, use of 874 LCFA carbon-13 tracers to measure plasma LCFA oxidation from expired [¹³C]-carbon 875 dioxide enrichment requires correction for possible retention of the carbon-13 label in the 876 exchange or transamination reactions that occur with tricarboxylic acid (TCA) cycle 877 878 intermediates (Sidossis et al. 1995a, 1995b). To this end, the acetate correction factor was 879 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 880 881 sodium acetate (Sidossis et al. 1995b). When the acetate correction factor was derived during 90 min of cycling at 60% VO₂max in a small sample (n = 5) of eumenorrheic women, 2-3 h 882 post prandial, during the EF, LF, and ML phases it was found to be modestly lower in the ML 883 (0.57) than EF (0.59) phase despite no difference in whole-body substrate utilisation 884 (Oosthuyse et al. 2003). While application of the correction factor would increase absolute 885 886 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 887 menstrual phases. Nevertheless, the greater carbon-label fixation in TCA cycle exchange 888 889 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. 890

891

892 Mechanisms behind menstrual cycle effects on protein metabolism

893 Increased protein catabolism in the ML phase

Lamont et al. (1987) were first to report greater total urea nitrogen excretion (a sum of urine 894 and sweat urea nitrogen) in the ML versus EF phase in recreationally active women measured 895 over 3 days that included a 60 min cycling exercise on day 1, indicating greater protein 896 catabolism in the ML phase. According to these findings, protein requirements per day in EF 897 were notably less than in the ML phase (1.05 g/kg vs. 1.36 g/kg per day, respectively). In 898 899 agreement, amino acid isotopic tracer studies in eumenorrheic women have consistently 900 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 901 902 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 903 904 menstrual cycle phase on plasma amino acid oxidation rate (Fig. 8a) but no clear effect on 905 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 906 907 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 908 occasions over several consecutive menstrual cycles in each participant and the rate of lysine 909 910 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 911 support protein synthesis. Considering that lysine oxidation was maintained at a higher rate at 912 all ingestion doses in ML versus EF phase, suggests the higher lysine requirement in ML 913 phase is due to higher amino acid catabolism (Kriengsinyos et al. 2004). 914

915

insert Figure 8 near here

917	Greater protein catabolism during the ML phase appears to be a P4 dominant effect because
918	when men received oral E2 supplements (2 mg/day) for 8 days to mimic plasma E2
919	concentration during the LF phase (876 pmol/L), leucine oxidation was notably decreased at
920	rest (PL: 28 \pm 2 vs E2: 23 \pm 2 $\mu mol/kg \cdot h)$ and during 90 min of exercise at 65% VO ₂ max (PL: 28 \pm 2 vs E2: 23 \pm 2 $\mu mol/kg \cdot h$) and during 90 min of exercise at 65% VO ₂ max (PL: 28 \pm 2 $\mu mol/kg \cdot h$) and during 90 min of exercise at 65% VO ₂ max (PL: 28 \pm 2 $\mu mol/kg \cdot h$) and during 90 min of exercise at 65% VO ₂ max (PL: 28 \pm 2 $\mu mol/kg \cdot h$) and during 90 min of exercise at 65% VO ₂ max (PL: 28 \pm 2 $\mu mol/kg \cdot h$) and during 90 min of exercise at 65% VO ₂ max (PL: 28 \pm 2 $\mu mol/kg \cdot h$) and during 90 min of exercise at 65% VO ₂ max (PL: 28 \pm 2 $\mu mol/kg \cdot h$) and during 90 min of exercise at 65% VO ₂ max (PL: 28 \pm 2 $\mu mol/kg \cdot h$) and during 90 min of exercise at 65% VO ₂ max (PL: 28 \pm 2 $\mu mol/kg \cdot h$) and during 90 min of exercise at 65% VO ₂ max (PL: 28 \pm 2 $\mu mol/kg \cdot h$) and during 90 min of exercise at 65% VO ₂ max (PL: 28 \pm 2 $\mu mol/kg \cdot h$) and during 90 min of exercise at 65% VO ₂ max (PL: 28 \pm 2 $\mu mol/kg \cdot h$) and during 90 min of exercise at 65% VO ₂ max (PL: 28 \pm 2 $\mu mol/kg \cdot h$) and during 90 min of exercise at 65% VO ₂ max (PL: 28 \pm 2 $\mu mol/kg \cdot h$) and during 90 min of exercise at 65% VO ₂ max (PL: 28 \pm 2 $\mu mol/kg \cdot h$) and during 90 min of exercise at 65% VO ₂ max (PL: 28 \pm 2 $\mu mol/kg \cdot h$) and during 90 min of exercise at 65% VO ₂ max (PL: 28 \pm 2 $\mu mol/kg \cdot h$) and during 90 min of exercise at 65% VO ₂ max (PL: 28 \pm 2 $\mu mol/kg \cdot h$) and during 90 min of exercise at 65% VO ₂ max (PL: 28 \pm 2 $\mu mol/kg \cdot h$) and during 90 min of exercise at 65% VO ₂ max (PL: 28 \pm 2 $\mu mol/kg \cdot h$) and during 90 min of exercise at 65% VO ₂ max (PL: 28 \pm 2 $\mu mol/kg \cdot h$) and during 90 min of exercise at 65% VO ₂ max (PL: 28 \pm 2 $\mu mol/kg \cdot h$) and during 90 min of exercise at 65% VO ₂ max (PL: 28 \pm 2 $\mu mol/kg \cdot h$).
921	62 ± 5 vs E2: 53 ± 4 $\mu mol/kg\cdot h)$ (Hamadeh et al. 2005). This resulted in a less negative
922	protein balance after E2 supplements compared with placebo. However, leucine flux and the
923	rate of protein synthesis were not altered by E2 supplements.

The mechanism whereby P4 upregulates and E2 downregulates protein catabolism remains 925 926 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 927 supplementation to mimic the LF phase (Hamadeh et al. 2005), E2 supplementation did not 928 alter skeletal muscle expression of the amino acid transamination enzyme, aspartate 929 aminotransferase, or the rate-limiting enzyme for branched-chain amino acid oxidation, 930 branched-chain 2-oxo-acid dehydrogenase (BCOAD), or its negative regulator, BCOAD 931 kinase (Fu et al. 2009). However, skeletal muscle BCOAD kinase mRNA content was 1.2-932 933 1.3-fold greater in women than men at rest (Fu et al. 2009), which in its role as a negative 934 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 935 (Tarnopolsky 2008). In that same study, skeletal muscle mRNA content of BCOAD kinase in 936 937 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 938 modest differences in ovarian hormone concentrations between phases suggesting that the 939

measurement in the luteal phase may have corresponded with the late luteal phase (Fu et al. 940 2009). Future studies should investigate the effect of P4 on these gene targets. Furthermore, 941 whether the ovarian hormones regulate skeletal muscle protein content or activity of 942 transamination and branched-chain amino acid oxidation enzymes remains to be determined. 943 Alternatively, some suggest P4 upregulation and E2 downregulation of thyroid function and 944 availability of free triiodothyronine (T3) (Ben-Rafael et al. 1987; Torre et al. 2020) is 945 946 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 947 948 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 949 protein, thyroid-binding globulin to maximise free T3 availability (Torre et al. 2020; 950 951 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 952 follicular phase. 953

954

The estimated average daily requirement of protein for female endurance athletes derived 955 from 3-day nitrogen balance measurements in two studies conducted in the MF phase in 956 female cyclists training 13 and 11 h/week is between 1.28 and 1.63 g/kg per day with high 957 958 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 959 findings, recommended daily intake (RDI) of protein for female endurance athletes has been 960 suggested as 1.6-2.0 g/kg per day, to allow for 12% interindividual variability (Mercer et al. 961 2020) but does not include menstrual phase specificity, which warrants further validation. 962

964 No menstrual phase effect on muscle protein synthesis

965	Only one study in eumenorrheic women has quantified the rate of muscle protein synthesis
966	between menstrual phases. In this study, unilateral leg exercise was performed with 1-legged
967	kicks and 1-leg acting as a resting control in the EF and ML phases (Miller et al. 2006).
968	Twenty-four hours later, fractional protein synthetic rate (FSR) was measured from a 4 h
969	constant infusion of [1- ¹³ C]leucine followed by a muscle biopsy to measure the percentage
970	uptake of the label into muscle myofibrillar protein in each leg. While FSR was higher in the
971	exercised leg 24h-post exercise compared with the resting leg, there was no menstrual phase
972	differences in resting (EF: 0.053±0.009%/h vs ML: 0.055±0.013%/h) or 24h-post exercise
973	(EF: 0.131±0.018%/h vs ML: 0.134±0.018%/h) FSR (Miller et al. 2006).
974	
975	Nevertheless E2-deficiency, as occurs in menopause, is known to promote muscle atrophy by
976	increased autophagic signalling through FOXO3 that is supressed by E2 replacement, where
977	E2 replacement increases muscle mass and strength further when combined with exercise
978	training possibly by regulation of satellite cell activity (Hansen, 2018). It may be that while
979	menstrual phase does not influence resting or recovery muscle protein synthesis in
980	eumenorrheic women, E2 could enhance exercise-stimulated anabolism by the activation and
981	proliferation of muscle satellite cells following exercise in a menstrual phase specific manner
982	as presented below.

983

984 Menstrual phase influences muscle regeneration and strengthening

985 Enns and Tiidus (2008) have clearly demonstrated these effects in OVX rodents receiving E2
986 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).

994

Interestingly, E2 influence over the satellite cell response to EIMD is more noticeable during 995 996 the MF compared with ML phase as shown by Haines et al. (2018) after unilateral eccentric knee extensor exercise. These authors found ER α muscle content and the increase in ER-997 DNA binding after exercise to be greater in the MF than ML phase (Haines et al. 2018). 998 Accordingly, the increase in expression of Myo-D, reflecting satellite cell activation, 999 1000 appeared greater and cyclin D1 reflecting mitotic cycling was significantly greater in the MF 1001 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 1002 1003 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 1004 1005 ER and satellite cell activity occurred independent of a difference in muscle E2 concentration 1006 and suggests that P4 in the ML phase suppressed E2 promotion of satellite cell differentiation 1007 and muscle regeneration after EIMD, possibly by downregulation of ER expression and 1008 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 1009 1010 and as such decreased plasma membrane disruption and EIMD in ML may also contribute to 1011 a reduced need for satellite cell activation and muscle repair in ML than EF phase.

1013 Consequently, a study that performed unilateral leg resistance training with 1-leg trained only 1014 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 1015 1016 than the luteal phase trained-leg (Sung et al. 2014). This occurred coincident to an increase in 1017 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. 1018 1019 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 1020 than luteal phase (Reis et al. 1995; Wikström-Frisén et al. 2017), with only a single report of 1021 no difference in outcomes after follicular phase-based versus luteal phase-based arm 1022 1023 resistance training at a lower frequency (1-3 d/week) (Sakamaki-Sunaga et al. 2016). 1024 Therefore, muscle regeneration and strengthening are mostly greater during the follicular 1025 phase when E2 is present alone (even in low-moderate concentrations) without P4.

1026

1027 Conclusion and perspectives

1028 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 1029 1030 phenolic structure stabilizes membranes and quenches free radicals, while genomic effects of 1031 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 1032 1033 EIMD response. Secondly, E2 promotion of skeletal muscle glucose uptake is not realized in 1034 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 1035

1036 efforts in carbohydrate-depleted states. For this reason, carbohydrate supplements during exercise are imperative in these menstrual phases and future studies should explore menstrual 1037 1038 phase effects when ingesting multiple-transportable carbohydrates at recommended doses during exercise, as well as variability in efficacy of ingesting exogenous glucogenic sources, 1039 1040 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 1041 1042 normal daily carbohydrate intakes should be considered for recovery or when planning preevent nutrition that will necessitate higher carbohydrate-loading intakes to equalize glycogen 1043 1044 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 1045 regulators of substrate partitioning, such that a menstrual phase increase in whole-body fat 1046 oxidation is only realized in the ML (or LF) phase under carbohydrate-restricted exercise and 1047 1048 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, 1049 1050 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 1051 should be explored. In particular, assessment of menstrual phase differences in protein 1052 1053 catabolism during exercise should be investigated and the possibility of recommending 1054 ingestion of rapidly absorbed peptides during exercise or more rigorous protein recovery 1055 supplements in the ML should be considered. Moreover, the molecular signaling mechanism 1056 explaining the P4 dominant effect promoting protein catabolism should be investigated. Lastly, athletes including adjunct strength training should periodize these training sessions in 1057 1058 the follicular phase to benefit from greater muscle strength gains owing to E2 activation of 1059 satellite cells that is supressed by P4 in the luteal phase. Following the evidence presented 1060 herein, this review serves to guide researchers in conceptualizing future studies where

- 1061 research is lacking, and to test progressive interventions for female athletes to circumvent or
- 1062 gain advantage from the menstrual phase-specific responses.

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1068 Author contributions

- 1069 TO, JAS, and ACH, conceived the review design; TO drafted the manuscript; TO, JAS, and
- 1070 ACH edited, revised, and approved the final version of the manuscript.
- 1071

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1601 **Figure captions**

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

1604

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

1608

1609 Fig. 3 Diagram showing the receptor-activated signaling pathways of estrogen (E2) and 1610 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 1611 1612 progesterone receptors (PR) in dimer complexes that translocate to the nucleus to regulate 1613 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 1614 1615 transactivate or trans-repress target gene expression. In the same way, E2 regulates 1616 mitochondrial DNA expression. The indirect genomic and rapid non-genomic effects (b) occur via binding plasma membrane-bound receptors complexed with G-proteins, namely, 1617 1618 ER and PR or alternate membrane receptors, G-protein coupled estrogen receptor (GPER) 1619 and progesterone receptor membrane complex 1 (PGRMC1), membrane progestin receptors 1620 (mPRs), respectively. On E2 and P4 binding, these membrane-bound receptors activate 1621 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 1622 1623 non-genomic cellular responses and membrane-bound ER signaling regulates endoplasmic/sarcoplasmic reticular calcium (Ca^{2+}) ion channel activity to increase cytosolic 1624

1625 Ca²⁺ content and related signaling. p38MAPK, p38 mitogen activated protein kinase;

1626 ERK1/2, extracellular signal-related kinases1/2, PI3K, phosphoinositide-3-kinase; Akt, RAC

1627 (Rho family)-alpha serine/threonine-protein kinase (also known as protein kinase B); AMPK,

1628 AMP-activated protein kinase

1629

Fig. 4 Diagram showing E2 and P4 signaling to influence exercise carbohydrate metabolism. 1630 In skeletal muscle, E2 transactivates or trans-represses GLUT4 gene expression via ERa or 1631 ER β , respectively, that bind specific DNA-bound transcription factors (1). GLUT4 1632 translocation to the plasma membrane is promoted by E2 independent of insulin via 1633 membrane-bound ERa that directly stimulates phosphoinositide-3-kinase (PI3K), which then 1634 1635 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 1636 1637 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 1638 degradation and thereby trans-represses expression of gluconeogenic target genes (4). 1639 Conversely, in a glucose-deprived state typical of fasted exercise, P4 trans-activates 1640 1641 expression of these gluconeogenic genes via binding membrane-bound PGRMC1 that 1642 activates adenylate cyclase and cyclic-AMP production that activates protein kinase A (PK-1643 A) to phosphorylate and activate the transcription factor, cAMP-response element binding 1644 protein (CREB) (5). CEBPA, CCAAT/enhancer-binding protein alpha; G6Pc, glucose-6phosphotase; IRS, insulin receptor substrate; NFκB, nuclear factor kappa-B; PDK1, 1645 phosphoinositide-dependent protein kinase-1; PEPCK, phosphenolpyruvate-carboxykinase; 1646 PIP3, PI-3,4,5-triphosphate; Rab, Rab-GTPase; SP-1, specific protein-1; TBC1D1/4, 1647 1648 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

1655 large effects, respectively

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1654

1657 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 1658 1659 bottom right, E2 bound ER α activates gene expression of the nuclear hormone receptors, 1660 PPARα and PPARδ directly or indirectly via phosphorylation of AMPK. PPARs enhance expression of plasma membrane (fatty acid transport protein, FATP and fatty acid 1661 1662 translocase, CD36), cytosolic (fatty acid binding protein cytosolic, FABPc) and mitochondrial (carnitine palmityltransferase 1, CPT1) LCFA transporters, beta oxidation 1663 enzymes (medium-chain acyl-CoA dehydrogenase, MCAD; trifunctional protein-alpha, 1664 TFP α ; β -hydroxy-acyl-CoA dehydrogenase, β -HAD) and kinases (pyruvate dehydrogenase 1665 1666 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 1667 localized lipoprotein lipase (LPL) to support plasma very low density lipoprotein triglyceride 1668 (VLDL-TG) hydrolysis for LCFA uptake, second by increasing the expression of the 1669 transcription factor, sterol regulatory element-binding protein 1c (SREBP1c) and downstream 1670 1671 targets for skeletal muscle IMTG storage (mitochondrial glycerol-3-phosphate acyl 1672 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 1673 depicted on the top left, by decreasing adipose-localized LPL activity, decreasing lipid 1674 storage by repressing SREBP1c expression and promoting adipose lipolysis by increasing β-1675 adrenergic receptor (β -AR) sensitivity to epinephrine, decreasing α_2 -AR sensitivity and 1676 directly or indirectly increasing perilipin activity. P4 antagonizes E2 effects possibly by 1677 1678 repressing ER expression

1679

Fig. 7 Forest plot showing the mean difference (95% confidence interval, CI) of menstrual 1680 phase on rate of plasma LCFA appearance (Ra), uptake (Rd), and oxidation (Rox) during 1681 exercise (a) and rate plasma glycerol appearance (Ra), and uptake (Rd) during exercise as an 1682 1683 indication of whole body lipolytic flux (b); showing the heterogenicity indices, z-scores, pvalues, and effect size (ES) for each outcome. Effect size scores are interpreted as: 0.0-0.2 is 1684 trivial; 0.2-0.6 is small; 0.6-1.2 is moderate; >1.2 is large effects, respectively 1685

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Fig. 8 Forest plot showing the mean difference (95% confidence interval, CI) of menstrual 1687 1688 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 1689 1690 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 1691 is trivial; 0.2-0.6 is small; 0.6-1.2 is moderate; >1.2 is large effects, respectively 1692 1693

Table 1 Reference ranges* for serum 17-β-estradiol (E2) and progesterone (P4)

1695	concentrations	that define	the menstrual	phases
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Menstrual	Recommended	Serum E2		Serum P4		Evidence
phase	timing	(pmol/L)		(nmol/L)		of
						ovulation
		Range of	Range of	Range of	Range of	
		Median	95%CI	Median	95%CI	
		values	values	values	values	
EF	days 1-7 from	129-	58-	0.6-	0.3-	
	onset of	154	225	1.3	3.8	
	menses					
MF	8 - 4 days	163-	113-	0.3-	0.3-	
	before the day	363	491	0.6	1.6	
	of ovulation					
LF	0 - 2 days	651-	337-	0.6-	0.3-	
	before the day	939	1518	2.5	4.1	
	of ovulation (or					
	LH surge)					
ML	5 -10 days after	451-	267-	32.4-	22.4-	Require a
	ovulation	552	807	42.5	60.4	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