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- 1 Effect of eccentric-based resistance exercise on bone (re)modelling markers across the 2 menstrual cycle and oral contraceptive cycle. 3 Isabel Guisado-Cuadrado¹, Nuria Romero-Parra^{1,2}, Rocío Cupeiro¹, Kirsty J. Elliott-Sale³, 4 Craig Sale³ & Ana B. Peinado¹ 5 1. LFE Research Group. Department of Health and Human Performance. Faculty of 6 Physical Activity and Sport Sciences. Universidad Politécnica de Madrid, Madrid, Spain. 7 2. Department of Physical Therapy, Occupational Therapy, Rehabilitation and Physical 8 Medicine. Faculty of Health Sciences. Universidad Rey Juan Carlos, Alcorcón, Spain. 9 3. Department of Sport and Exercise Sciences, Manchester Metropolitan University 10 Institute of Sport, Manchester, UK. 11 12 ORCID and e-mail: 13 Isabel Guisado-Cuadrado: 0000-0002-1767-5341 / i.guisadoc@upm.es 14 Nuria Romero-Parra: 0000-0001-9754-5565 / nuria.romero@urjc.es 15 Rocío Cupeiro: 0000-0002-4119-0002 / rocio.cupeiro@upm.es 16 Kirsty J. Elliott-Sale: 0000-0003-1122-5099 / K.Elliott-Sale@mmu.ac.uk 17 Craig Sale: 0000-0002-5816-4169 / C.Sale@mmu.ac.uk 18 Ana B. Peinado: 0000-0002-4871-8682 / anabelen.peinado@upm.es 19 20 Corresponding author: 21 Isabel Guisado-Cuadrado 22 LFE Research Group. Department of Health and Human Performance. Faculty of Physical 23 Activity and Sport Science. Universidad Politécnica de Madrid 24 Calle de Martín Fierro, 7, 28040 Madrid, España
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27 Abstract

Purpose: To investigate the acute effects of eccentric-based resistance exercise and sex
hormone fluctuations on P1NP and β-CTX-1 concentrations in premenopausal females.
Methods: Nine eumenorrheic females and ten oral contraceptive (OC) users performed
eccentric-based resistance exercise, consisted of 10x10 repetitions of parallel back
squats with a 4-second eccentric phase, in the early-follicular (EFP), late-follicular (LFP)
and mid-luteal (MLP) phases of the menstrual cycle (MC) or in the withdrawal (WP) and
active pill-taking (APP) phases of the OC cycle.

- 35 Results: 17β-oestradiol (pg·ml-1) was lower in EFP (36.63±29.93) compared to LFP 36 (224.81±233.81;p=<0.001) and MLP (161.45±110.08;p<0.001) and higher in WP 37 (24.857±29.428) compared to APP (12.72±13.36;p=0.004). Progesterone (ng·ml-1) was 38 higher in MLP (8.30±5.23) compared to EFP (0.33±0.33;p<0.001) and LFP 39 (0.21±0.18;p<0.001), no significant differences were observed between the WP and 40 APP. In eumenorrheic females, β -CTX-1 (ng·ml-1) was lower in MLP (0.395±0.126) 41 compared to LFP (0.472±0.137;p=0.044). Comparing MC vs OC phases, eumenorrheic 42 females had higher P1NP levels (ng·ml-1) compared to OC users: EFP (62.54±13.13) vs 43 APP (50.69±8,91;p=0.034), LFP (67.32±18.96) vs WP (52.16±10.72; p=0.047), LFP vs APP 44 (p=0.025), MLP (67.51±19.34;p=0.049) vs WP, MLPvsAPP (p=0.027). Exercise time effect 45 showed lower β-CTX-1 concentrations 2h post-exercise (MC: 0.376±0.114,p<0.001; OC: 46 0.340±0.156,p=0.030) compared to pre-exercise (MC: 0.485±0.137; OC: 0.428±0.188) in 47 all participants.
- 48 Conclusions: β-CTX-1 concentrations were lower in the mid-luteal phase, emphasizing
 49 the importance of standardizing bone marker measurements to a specific MC phase. OC
 50 users exhibited reduced P1NP levels, underscoring the need to investigate synthetic and
 51 endogenous hormones' impact on long-term bone structure and strength.
- 52

53 Trial registration

54 The study was registered at Clinicaltrials.gov NCT04458662 on 2 July 2020.

- 56 **Keywords:** oestradiol; progesterone; sex hormones; P1NP; β-CTX; training
- 57

58 Abbreviations

P1NP	Procollagen type I N-propeptide
β-CTX-1	Cross-linking telopeptide of type I
	collagen
DXA	Dual-energy X-ray absorptiometry
OC	Oral contraceptive
MC	Menstrual cycle
WP	Withdrawal phase
АРР	Active pill-taking phase
EFP	Early follicular phase
LFP	Late follicular phase
MLP	Mid luteal phase
1RM	1-repetition maximum
SHBG	Sex hormone binding globulin
SD	Standard deviation

60 Introduction

61 Exercise guidelines to improve bone strength generally recommend exercises 62 that transmit both ground and joint reaction forces (e.g., impact and resistance-based 63 modalities) (Beck et al. 2017). Therefore, exercise provides a stimulus for bone tissue, 64 hence the analysis of procollagen type I N-propeptide (P1NP), as a biomarker of bone 65 formation, and cross-linking telopeptide of type I collagen (β-CTX-1), reflecting bone 66 resorption, may elucidate how exercise can affect bone metabolism given that 67 traditional techniques [dual-energy X-ray absorptiometry (DXA), computed tomography 68 or magnetic resonance imaging] are slow to respond to stimuli, and measurable changes 69 take months or even years to occur (Hart et al. 2020; Eriksen 2010). Bone biomarker 70 measurements are frequently used to provide insight into the bone response to acute 71 exercise interventions (Dolan et al. 2022); specifically, they provide information about 72 physiological alterations in bone metabolism, such as the prevalence of formative or 73 resorptive activity (Hart et al. 2020). A recent meta-analysis (Dolan et al. 2022) shows 74 that the typical bone response to an acute bout of exercise is an increase bone 75 resorption and formation markers. Nevertheless, the circulating biomarkers response 76 depends on the exercise type and impact loading, leading to highly variable outcomes 77 (Dolan et al. 2022). Insufficient data were available to evaluate response to resistance 78 exercise while bone resorption biomarkers showed no response (Dolan et al. 2022). 79 However, sample timing is important as CTX-1 peaked within 2 h post-exercise (Dolan et 80 al. 2022).

81 This relationship between exercise and bone remodelling becomes even more 82 complex when considering the role of sex hormones. Beyond its reproductive function, 83 17β-oestradiol is also a key regulator of bone metabolism (Khosla and Monroe 2018). In 84 fact, in vitro studies have shown that the oestrogen receptor is involved in the 85 osteogenic response to mechanical stress, thus low concentrations of 17β-oestradiol 86 could reduce the mechanosensitivity of osteocytes and the responsiveness of bone cells 87 to mechanical load (Riggs et al. 2002; Windahl et al. 2013; Klein-Nulend et al. 2015). In 88 addition, progesterone has been shown to stimulate osteoblast differentiation in vitro 89 (Seifert-Klauss and Prior 2010). The female menstrual cycle (MC) is characterized by 90 fluctuating 17β-oestradiol and progesterone (Oosthuyse et al. 2022), although not all 91 females follow these patterns of hormonal fluctuations, given that there is a large

92 proportion of the athletic population that uses oral contraceptives (OC) (Martin et al. 93 2018). During the hormonal active pill-taking phase (APP) 17α -ethinyl oestradiol inhibits 94 endogenous 17-β-oestradiol production, while in the placebo or withdrawal phase (WP), 95 endogenous 17β -oestradiol increases again (Willis et al. 2006). Based on this theoretical 96 justification, there is in vivo evidence on the effect of sex hormones on bone remodelling 97 markers. Some investigations show that CTX-1 is lower during the luteal phase of 98 ovulating females compared to the follicular phase (Mozzanega et al. 2013; Gass et al. 99 2008; Guisado-Cuadrado et al. 2024), and P1NP higher during luteal phase (Gass et al. 100 2008), although results remain inconsistent, as some studies show no differences 101 between phases (Guzman et al. 2022). In turn, CTX-1 has been found to be lower in the 102 APP compared to the WP (Martin et al. 2021). And when comparing ovarian hormonal 103 profiles, OC users during the APP have lower P1NP and CTX-1 concentrations compared 104 to eumenorrheic/non-OC users females (He et al. 2022; Guisado-Cuadrado et al. 2024; 105 Glover et al. 2009). Considering this evidence, it is important to investigate whether 106 different endogenous sex hormone concentrations could affect circulating 107 concentrations of bone (re)modelling markers, both at rest and in response to resistance 108 training.

Therefore, attending the close relationship between bone metabolism and ovarian sex hormones, this study aimed to examine the bone (re)modelling marker concentrations in eumenorrheic females and OC users at rest and in response to resistance training across the different phases of the MC and OC cycle.

113 Methods

114 Participants

115 Participants included in this study were a subsample selected from the 116 participants enrolled in the IronFEMME project (Peinado et al. 2021). The purpose of 117 IronFEMME was to determine the influence of sex hormones on iron metabolism and 118 muscle damage, hence, the present study is a secondary analysis that was carried out 119 after the trial was completed. This trial was registered at clinicaltrials.gov 120 (NCT04458662). To be included in the IronFEMME study, participants were required to 121 meet the following criteria: (i) healthy adult females between 18 and 40 years; (ii) 122 regular MCs (defined as normally occurring MCs from 21 to 35 days in length) (Elliott-

123 Sale et al. 2021) at least 6 months prior to the study ; (iii) or using monophasic combined 124 OC pills for at least 6 months prior to the study; (iv) no regular consumption of 125 medication or nutritional supplements; (v) non-smokers; (vi) non-pregnant; and (vii) 126 experienced in resistance training performing at least 30 min session two times per week 127 during a minimum of a year. The present trial was performed as a secondary analysis 128 using serum samples collected and frozen from the IronFEMME project. Participants 129 from the IronFEMME project were further selected for inclusion in the current analysis 130 according to: (i) aged between 20 and 30 years; (ii) bone injury free for the at least 12 131 months or muscle injury free for at least 6 months. Following this further selection, the 132 data from nine eumenorrheic females and ten monophasic OC users (see Table 1 for 133 participants' characteristics and training volume) were included in the current analysis. 134 Participants received ethical clearance from the Research Ethics Committee of the 135 Universidad Politécnica de Madrid and were informed of the study procedures (*i.e.*, for 136 the present study on bone (re)modelling) and risks prior to participation and written 137 informed consent was obtained from each subject prior to inclusion. Participants also 138 agreed to the use of their data for other scientific purposes a posteriori.

139

140 *MC and OC cycle monitoring*

141 The protocols used for MC and OC cycle monitoring have been previously described 142 (Peinado et al. 2021; Guisado-Cuadrado et al. 2024). In brief, for the MC group, 143 menstruation, ovulation, and mid-luteal progesterone levels were established using 144 gold-standard techniques (Elliott-Sale et al. 2021). Finally, MC phases were verified using 145 blood samples taken on each of the eccentric testing days. The EFP was characterised 146 by lower levels of 17β -oestradiol and progesterone. The LFP was characterised by higher 147 17β-oestradiol concentrations than in the EFP and MLP and higher progesterone 148 concentrations than in the EFP, but lower than 6.36 nmol/L. The MLP was characterised 149 by a progesterone concentration greater than 16 nmol/L and 17β-oestradiol higher than 150 in the EFP but lower than in the LFP.

OC users took their active hormone pill daily for 21 days during the APP, followed by a 7-day WP (pill without hormonal content). Endogenous sex hormone concentrations were analyzed in serum in each phase. The mean duration of the OC use was 3.9±3 years (mean±SD). The brands and dosages of exogenous sex hormones in the

155 monophasic combined OC preparations used by these participants were as follows: 156 Yasmin[®] (n=2): 0.03 mg ethinyl oestradiol and 3 mg drospirenone; Linelle[®] (n=1): 0.02 157 mg ethinyl oestradiol and 0.1 mg levonorgestrel; Sibilla[®] (n=1): 0.03 mg ethinyl 158 oestradiol and 2 mg dienogest; Yasminelle[®] (n=1): 0.02 mg ethinyl oestradiol and 3 mg 159 drospirenone; Levobel[®] (n=1): 0.02 mg ethinyl oestradiol and 0.10 mg levonorgestrel; 160 YAZ[®] (n=1): 0.02 mg ethinyl oestradiol and 3 mg drospirenone; Diane 35[®] (n=1): 0,035 161 mg ethinyl oestradiol and 2 mg ciproterone; and Loette[®] (n=2): 0.02 mg ethinyl 162 oestradiol and 0.1 mg levonorgestrel.

163

164 Experimental overview

165 Eumenorrheic participants came to the laboratory on four occasions, the first one to 166 perform a 1-repetition maximum (1RM) test and the following three times (Figure 1) to 167 perform an eccentric-based resistance exercise in each of the MC phases evaluated (EFP, 168 LFP and MLP). Testing sessions took place on cycle days 4±1 for the EFP, 12±3 for the 169 LFP and 23±2 for the MLP. The LFP testing session was arranged 2 days prior to estimated 170 LH surge, which was based upon retrospective cycles' LH surge confirmation. If LH peak 171 was not observed during the 2 subsequent days to LFP testing session, this trial was 172 considered invalid. OC users came to the laboratory on 3 occasions, the first visit for the 173 1RM test and the following two occasions to carry out the eccentric-based resistance 174 exercise on days 5±2 and 13±2 of WP and APP the OC cycle (Figure 1). This study 175 analysed only the early pill-taking phase (first week after the first pill); however, it should 176 be noted that exogenous sex hormone concentrations increase over the days of pill-177 taking (Willis et al. 2006). 24 h prior to all laboratory visits, all participants were 178 instructed to refrain from alcohol, caffeine, and any intense physical activity or sport. 179 Cycle phases order to perform the eccentric-based exercise protocol was randomized 180 and counterbalanced for both eumenorrheic and OC participants.

181

182 1 RM estimation

On screening day, volunteers attended the laboratory between 8:00 a.m. and 10:00 a.m. in a resting and fasted state during the EFP in the eumenorrheic group and day 4-7 of the WP in the OC users. Baseline antecubital venous blood samples were collected for complete blood count, biochemical, and hormonal analysis. After collecting

187 the blood sample, a total body DXA was performed. The 1RM of the parallel back-squat 188 exercise was estimated by using the Powerlift App (Carlos Balsalobre-Fernández, 189 Madrid, Spain) (Balsalobre-Fernández et al. 2017), based on the force load-velocity 190 relationship (González-Badillo and Sánchez-Medina 2010). This app has been proved to 191 be highly valid, reliable, and accurate for the measurement of barbell velocity in the 192 squat exercise (Balsalobre-Fernández et al. 2017). Participants performed an 193 standardized warm-up (Peinado et al. 2021). After that, the test consisted of 4 sets of 1 194 repetition with submaximal loads proportionally increased between 70% and 90% of 195 participants' maximum self-reported. To record the videos, a researcher (always the 196 same) held an iPhone 6S (Apple Inc., Cupertino, CA, USA) in portrait position and 197 recorded each lift with a high-speed camera (240 Hz) (see the detailed methodology in 198 Peinado et al. (Peinado et al. 2021)).

199

200 Eccentric-based resistance exercise

201 After 1RM estimation, the eccentric-based resistance exercise sessions were 202 performed based upon the obtained values. The exercise protocol consisted of 10 sets 203 of 10 reps of plate-loaded barbell parallel back squats, at 60% of their 1RM, with 2 min 204 of rest between sets. Squats were performed at a tempo of 4-seconds eccentric 205 movement, 1-s pause at the bottom, 1-s concentric movement, and a 1-second pause 206 at the top of the lift. This protocol was designed for the IronFEMME project with the aim 207 of triggering muscle damage (MacDonald et al. 2014). Although, this work may extend 208 existing evidence, as the characteristics of the exercise protocol in this study differ from 209 others (Sherk et al. 2013; Rogers et al. 2011), as it focuses on the eccentric phase of 210 exercise.

211

212 Blood collection

Blood samples were taken between 8 and 11 a.m. to avoid diurnal variability of biochemical parameters (Szulc et al. 2017) and within a participant the timeframe was minimised to 1 hour within the 3 h total window in the different phases of the MC and OC to reduce the intra-participant variability of the results. Two samples (pre- and 2h post- eccentric-based resistance exercise) were drawn from each participant at each MC or OC phase, from an antecubital vein while they were seated to determine the bone 219 (re)modelling marker [procollagen type I N-propeptide (P1NP) and carboxy-terminal 220 cross-linking telopeptide of type I collagen (β -CTX-1)] and sex hormone (17 β -oestradiol 221 and progesterone) concentrations. Sex hormone binding globulin (SHBG) was measured 222 only at rest. All venous blood samples were obtained using a 21-gauge (0.8 mm × 19 223 mm, Terumo®) needle. Blood samples for serum variables were collected in a 9 mL Z 224 serum separator clot activator tubes (Vacuette®) and allowed to clot at room 225 temperature for 60 minutes. They were then centrifuged for 10 minutes at 1610 g to 226 obtain the serum (supernatant), divided into 600 μ L aliquots, and stored at -80°C.

227

228 Blood analysis

229 17 β -oestradiol, progesterone, SHBG, P1NP and β -CTX-1 were analysed in serum 230 by electrochemiluminescent immunoassay using Roche Diagnostics reagents in a Cobas 231 e411 Elecsys automated analyser (Roche Diagnostics GmbH, Mannheim, Germany) in 232 the Spanish National Centre of Sport Medicine (Madrid, Spain). Inter-assay and intra-233 assay CV were: 1.8 and 2.4% at 57.2 ng·ml⁻¹ level for P1NP; were 2.1 and 2.8% at 0.403 234 ng·ml⁻¹ level for β -CTX; 11.9% and 8.5% at 93.3 pg·ml⁻¹ and 6.8% and 4.7% at 166 pg·ml⁻¹ 1 for 17β-oestradiol; 23.1% and 11.8% at 0.7 ng·ml 1 and 5.2% and 2.5% at 9.48 ng·ml 1 235 236 for progesterone; and 2.4 and 2.8% at 44.2 nmol·l⁻¹ level and 2.7 and 5.6% at 204 nmol·l⁻ 237 ¹ level for SHBG.

238

239 Nutritional recommendations

A nutritionist prescribed the breakfast meal, and participants replicated the same breakfast at least 2h prior to the eccentric-based resistance protocol in all the MC and OC phases. Nutritional recommendations were standardised 48 h prior the eccentric-based resistance protocol (for diet composition see Supplementary Material 1).

245

246 Statistical analysis

Normality tests were performed using the Shapiro-Wilk test. 17β-oestradiol,
 progesterone and SHBG were non-normally distributed, thus, they were log transformed for analysis (Hackney and Viru 2008).

250 Participant characteristics were analysed using independent samples t-tests. 251 SHBG was compared between MC phases (EFP vs LFP vs MLP) using a one-way ANOVA 252 and OC cycle phases (WP vs APP) using a paired t-test. To compare SHBG between MC 253 vs OC phases an independent t-test was conducted for each comparison. Mean 254 concentrations of 17β-oestradiol, progesterone, P1NP and β-CTX-1 were compared 255 between MC phases and OC cycle phases using the mixed linear model to analyse 256 repeated measures. The phases and time were set as fixed effects (both intra-subject), 257 and participants were set as random effects. Comparing hormonal profiles, the mixed 258 linear model analysis was also performed, conducting a separate analysis for each of the 259 following comparisons: EFP vs WP, EFP vs APP, LFP vs WP, LFP vs APP, MLP vs WP, and 260 MLP vs APP. Ovarian hormonal profile (inter-subject) and time (intra-subject) were set 261 as fixed effects, and participants were set as random effects. Bonferroni's post hoc test 262 was applied to pairwise comparisons when the main effect was significant (p<0.05). The 263 ANOVAs effects sizes are reported as partial eta squared ($\eta^2 p$) whose interpretation is 264 0.01 = small, 0.06 = moderate, 0.14 = large effect. For pairwise comparisons Cohen's d 265 was used and interpreted based upon the following criteria: 0.2 = small, 0.5 = medium, 266 0.8 = large effect (Cohen 1992). Data are presented as mean±1SD.

267 Results

268 Sex hormones

269 Significant main effect of phase was observed for 17β-oestradiol in 270 eumenorrheic females, showing lower 17β-oestradiol levels in the EFP compared to the 271 LFP (p<0.001; d=-2.144) and MLP (p<0.001; d=-2.036) (Table 2). No main effect of time 272 or interaction was observed. While a significant main effect of phase was observed for 273 progesterone, where concentrations were significantly higher in the MLP compared to 274 the EFP (p<0.001; d=-2.840) and LFP (p<0.001; d=-3.194). No main effect of time or 275 interaction was observed (Table 2). SHBG concentrations at rest were not significantly 276 different between MC phases (see Table 2).

In OC users, significant main effect of phase was observed, where 17β-oestradiol
 concentrations were lower in the APP than in the WP. No main effect of time or
 interaction was observed (Table 2). Progesterone showed no main effect of phase, time,

or interaction (see on Table 2). SHBG concentrations at rest were not significantlydifferent between OC phases (see Table 2).

282 Comparing different ovarian hormone profiles, a significant main effect of 283 hormonal profile (MC vs OC) was observed for endogenous 17β-oestradiol in the 284 following comparisons: EFPvsAPP (F=8.288; p=0.010; η²p=0.328), LFPvsWP (F=25.322; 285 p<0.001; η²p=0.598), LFPvsAPP (F=51.870; p<0.001; η²p=0.753), MLPvsWP (F=30.173; 286 p<0.001; η²p=0.639), and MLPvsAPP (F=73.763; p<0.001; η²p=0.813), where 17β-287 oestradiol was higher in these MC phases compared to the OC phases indicated above 288 (see 17β-oestradiol concentrations in Tables 2). Progesterone concentrations showed a 289 significant main effect of hormonal profile for the following analyses: MLPvsWP 290 (F=34.120; p<0.001; n²p=0.667) and MLPvsAPP (F=32.288; p<0.001; n²p=0.655), where 291 progesterone concentrations were higher in the MLP compared to both OC phases (see 292 concentrations in Tables 2). SHBG concentrations were higher in OC phases compared 293 to MC phases: EFPvsWP (p<0.001; d=-2.078), EFPvsAPP (p<0.001; d=-2.561), LFPvsWP 294 (p=0.009; d=-1.355), LFPvsAPP (p=0.002; d=-1.732), MLPvsWP (p=0.022; d=-1.157) and 295 MLPvsAPP (p=0.004; d=-1.508) (see Table 2).

296

297 P1NP

298 No main effect of phase, time, or interaction (phase*time) within group was 299 shown (see Figure 2).

A significant main effect of hormonal profile was shown comparing MC vs OC phases, where EFP (F=5.329; p=0.034; $\eta^2 p$ =0.239), LFP (F=5.999; p=0.025; $\eta^2 p$ =0.261) and MLP (F=5.588; p=0.027; $\eta^2 p$ =0.257) reflected higher P1NP concentrations compared to APP, while LFP (F=4.580; p=0.047; $\eta^2 p$ =0.212) and MLP (F=4.516; p=0.049; $\eta^2 p$ =0.210) showed a higher concentration in comparison with WP (see Figure 2).

305

306 *B-CTX-1*

307 Significant main effect of phase was observed in eumenorrheic females (F=3.390; 308 p=0.044; $\eta^2 p$ =0.257), where serum concentrations were lower in the MLP compared to 309 LFP (p=0.044; d=0.617); and main effect of time (F=19.861; p<0.001; $\eta^2 p$ =0.871), 310 showing lower concentrations post-exercise (0.376±0.114 ng·ml⁻¹) than pre-exercise 311 (0.485±0.137 ng·ml⁻¹). No interaction (phase*time) was observed (see Figure 2). 312 Significant main effect of time in OC users was observed (F=5.224; p=0.030; 313 $\eta^2 p=0.445$), showing lower values post-exercise (0.340±0.156 ng·ml⁻¹) compared to pre-314 exercise (0.428±0.188 ng·ml⁻¹). No main effect of phase or interaction was observed.

315 It was observed a main effect of hormonal profile in LFP vs APP analysis, 316 reflecting higher β -CTX-1 concentrations in the LFP of the MC (F=14.181; p=0.040; 317 $\eta^2 p$ =0.225) (see Figure 2).

318 Discussion

319 This study is the first to investigate the effect of eccentric-based resistance 320 exercise on bone formation (P1NP) and resorption (β -CTX-1) markers in young 321 eumenorrheic females and OC users, within and between both ovarian hormonal 322 profiles. β -CTX-1 concentrations were significantly lower 2h after resistance exercise 323 without any change in P1NP, regardless of ovarian hormonal profile or phase. This acute 324 reduction in bone resorption may be succeeded by a reduction in bone formation, since, 325 based on the bone remodelling traditional theory, bone resorption and bone formation 326 processes are typically coupled (Heaney 1994). Nevertheless, it is unlikely that an acute 327 decrease in bone resorption represents the beginning of the cycle of bone remodelling 328 in response to exercise and is subsequently accompanied by a decrease in bone 329 formation, as combined reductions in bone resorption and bone formation are more 330 likely to occur under conditions of disuse (Hughes et al. 2020). What has been described 331 in the literature is an increase in serum markers of bone resorption after the onset of 332 mechanical loading, which is understandable considering the cellular response to this 333 new stimulus (Hughes et al. 2020). Although the relationship between acute change in 334 bone remodelling marker concentrations and long-term structural bone changes is not 335 yet well understood and future research should investigate the long-term potential for 336 a protective effect of eccentric resistance exercise on bone health, this study highlights 337 the need to understand the characteristics of resistance training for optimal bone health 338 in female athletes with different ovarian hormonal profiles.

339

The effect of resistance training on β-CTX-1 concentrations can only be directly
compared with the study by Rogers et al. (2011), as it measured β-CTX-1 concentrations
2 hours post-exercise. The significantly lower β-CTX-1 concentrations observed 2 hours

343 after the eccentric-based resistance exercise in the present study do not align with the 344 findings of Rogers et al. (2011), where no significant change in β -CTX-1 was reported 2 345 hours post-resistance exercise. Additionally, the present findings contradict the results 346 of Dolan et al. (2022) in their meta-analysis, where no biomarker response was observed 347 following a single session of resistance training. Nevertheless, it should be highlighted 348 the different effects of this eccentric-based exercise modality performed in the present 349 study, achieved by extending the eccentric phase over time (4 s eccentric movement), 350 in comparison with previous studies. Eccentric contractions have unique characteristics, 351 approximately 20-60% more force can be generated during eccentric contractions 352 compared to concentric contractions, this fact is highly relevant in explaining the acute 353 responses after exercise (Douglas et al. 2017). Given that muscle contraction is the main 354 source of mechanical loading that causes bone adaptations, because of the mechanical, 355 biochemical and molecular muscle-bone interplay (Brotto and Bonewald 2015), there is 356 a need to better understand the characteristics of resistance exercise training that may 357 further benefit bone health. In addition, it should be noted that none of these 358 investigations (Sherk et al. 2013; Rogers et al. 2011) included eumenorrheic females, so 359 it is unknown whether the influence of sex hormones may have affected the response 360 to resistance exercise in these studies. Given the timing of the exercise session and the 361 sample collection, it is possible that the lower β -CTX-1 concentrations observed 2 hours 362 post-exercise were influenced by the biomarker's typical circadian pattern. β -CTX-1 363 follows a diurnal rhythm, peaking around 5:00 a.m. and reaching its lowest levels by 364 approximately 2:00 p.m. (Szulc et al. 2017).

365 Comparing bone resorption and formation markers between phases of the MC, 366 β-CTX-1 was lower in the MLP compared to LFP, regardless of whether pre or post 367 exercise. As the 17β -oestradiol/progesterone ratio may be important in interpreting the 368 effect of the menstrual cycle, LFP was included in this study because 17β-oestradiol 369 concentrations are very high, and progesterone is very low compared to MLP. Thus, the 370 LFP may represent an ideal time to assess the effect of 17β-oestradiol with relatively low 371 progesterone concentrations. Whereas high 17β-oestradiol concentrations seem to 372 increase bone formation in vitro (Klein-Nulend et al. 2015; Windahl et al. 2013) and in 373 vivo models (Guisado-Cuadrado et al. 2024; Gass et al. 2008), progesterone seems to 374 reduce β -CTX-1 concentrations. These findings agree with previous studies (Guisado-

375 Cuadrado et al. 2024; Mozzanega et al. 2013; Gass et al. 2008), suggesting that a lower 376 17β-oestradiol/progesterone ratio may decrease β-CTX-1 concentrations. However, 377 these findings differ from those reported by Guzman et al. (2022), where no differences 378 were found between MC phases (mid-late follicular and luteal phases) following a 379 running protocol. Nonetheless, it should be noted that the Guzman et al. (2022) study 380 did not measure the LFP (1–3 days before ovulation, day 12 \pm 3), as defined in the 381 present study, but instead measured the mid-late follicular phase. The differences in 382 timing between the present study and Guzman et al. (2022) may explain the conflicting 383 results, as the participants in Guzman et al. (2022) had lower concentrations of 17β-384 oestradiol in the follicular phase than in the luteal phase, whereas in the present study, 385 17β -oestradiol levels were higher in the LFP compared to the MLP. Therefore, as 386 previously suggested in the literature (Hackney et al. 2022), the relationship between 387 17β-oestradiol and progesterone must be taken into account when interpreting the 388 effect of the MC.

389 Regarding the results from OC users, no differences in P1NP and β -CTX-1 levels 390 were observed between OC phases (see days in Fig. 1). This contrasts with the findings 391 of He et al. (2022), where β -CTX-1 concentrations were lower in the mid APP (days 22 to 392 28) and P1NP concentrations were lower in the mid and late APP (days 10 to 26) at rest. 393 Additionally, our results disagree with those of Martin et al. (2021), who found lower β -394 CTX-1 levels in the APP (days 15–16) compared to the WP (days 3–4) at rest. It is 395 important to highlight that the participants in the studies by He et al. (2022) and Martin 396 et al. (2021) used a specific OC formulation (30 μg ethinyl oestradiol and 150 μg 397 levonorgestrel), in contrast to the participants in our study, which may explain the 398 differences in results.

399 When MC and OC phases were compared, β -CTX-1 was lower in the APP 400 compared to the LFP. This finding is in line with other studies in which OC users were 401 shown to have significantly lower bone resorption marker levels (Glover et al. 2009; He 402 et al. 2022), suggesting an inhibition of bone metabolism. On the other hand, P1NP was 403 lower in the WP compared to the LFP and MLP of the MC, and in the APP compared to 404 all the MC phases. As mentioned above, 17β -oestradiol plays an important role in bone 405 metabolism by promoting bone formation (Klein-Nulend et al. 2015; Windahl et al. 406 2013). Over the course of the OC cycle, the concentration of endogenous 17β -oestradiol

407 in the APP was low, while in the WP it increases but remains low, similar to the EFP of 408 the MC (see Table 2). This low concentration of endogenous 17β-oestradiol may explain 409 the lower bone formation shown by OC users compared to eumenorrheic females, in 410 line with other investigations (Glover et al. 2009; He et al. 2022). Nevertheless, OC users 411 show a low concentration of endogenous sex hormones resulting from the negative 412 feedback effect of synthetic hormones on the anterior pituitary (Willis et al. 2006). 413 During APP the dose of synthetic hormones in these participants is 0.02-0.035 mg ethinyl 414 oestradiol. However, although this synthetic hormone shows a similar affinity for the 415 oestrogen receptor α as 17 β -oestradiol (Gutendorf and Westendorf 2001), other factors 416 may mediate the bioavailability of this hormone such as the low dose of synthetic 417 hormone contained in these OCs, the possible binding of progestins to the oestrogen 418 receptor α (Louw-Du Toit et al. 2017) or the significantly higher concentration of SHBG 419 in OC users compared to eumenorrheic females (see Table 2 and Results section). In fact, 420 other studies have already observed that ethinyl oestradiol (in OCs) has a dose-421 dependent stimulatory effect on hepatic SHBG production, leading to a reduction in 422 bioavailable 17β-oestradiol (Riggs et al. 2002). Notably, other studies have already 423 observed a negative association between SHBG and P1NP concentrations (Ackerman et 424 al. 2019). These results may provide some evidence for differences in bone metabolism 425 in women with different ovarian hormonal profiles, especially in altered bone formation. 426 However, as mentioned previously, these results need to be supported by long-term 427 studies to understand the effect of these bone markers on bone health. Although there 428 is already some evidence of an association between exposure to OCs and lower BMD 429 (Rocca et al. 2021; Guisado-Cuadrado et al. 2023).

430 The main strength of this study was the consideration of the hormonal 431 environments throughout the MC and OC cycle, measuring serum 17β-oestradiol and 432 progesterone, and using ovulation tests to measure LH surge according the most recent 433 guidelines (Elliott-Sale et al. 2021). Furthermore, exercise trials were performed in the 434 morning at the same time, using standardized protocols and indications (Szulc et al. 435 2017) for the preservation and measurement of serum sex hormones and bone 436 (re)modelling markers to avoid variability within and between subjects. The exercise 437 sessions were supervised by sports science professionals, which may help to

homogenise the stimulus achieved in each session by the participants. In addition, this
original research could expand the knowledge on bone metabolism and exercise, since
up to now most of the evidence refers to endurance (running or cycling) exercise in
males (Dolan et al. 2022), while the data to evaluate response to resistance exercise
were limited.

443 Methodological considerations

444 A specific type of OC with standardized composition and doses of synthetic 445 hormones was not used. Given the different properties of different synthetic progestins 446 in terms of binding affinities and transcriptional activities when binding to androgen or 447 oestrogen receptors, there could be a different magnitude of effect and biological 448 consequence (Louw-Du Toit et al. 2017). It should be mentioned that although 449 endogenous sex hormones have been measured in serum and OC doses have been 450 reported, in order to know the synthetic hormones bioavailability, ethinyl oestradiol and 451 progestin serum concentrations should have been measured. Another limitation that 452 may affect the interpretation of this study's results is that the β -CTX-1 marker exhibits 453 diurnal variability (Szulc et al. 2017). This limitation could have been addressed by 454 including a control group without exercise. Finally, the fact that samples were not taken 455 immediately after exercise may have meant that some transient changes were missed.

456 **Conclusion**

457 In conclusion, after 2h post eccentric-based resistance β -CTX-1 concentrations 458 were lower, regardless of ovarian hormonal status. This lower concentrations in β-CTX-459 1 do not seem to correspond to known physiological mechanisms triggered by exercise, 460 suggesting the need for further exploration to understand the mechanism that triggers 461 resistance training on bone metabolism. Analysing MC phases and OC use, our study 462 revealed an influence of hormonal fluctuations on bone (re)modelling markers. Lower 463 β -CTX-1 concentrations in the mid luteal phase suggesting that hormonal fluctuations 464 impact bone resorption throughout the MC. In addition, when ovarian hormonal profiles 465 were compared, OC users exhibited lower P1NP concentrations, emphasizing the 466 importance of investigating the role of synthetic hormones and endogenous sex 467 hormones in the potential long-term effects that these OCs may have on bone structure 468 and strength.

469

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477 Conflict of interest

- 478 The authors declare that they have no conflict of interest.
- 479

480 Ethics approval and consent to participate

- 481 This study complied with the amended Declaration of Helsinki and was approved by
- 482 Universidad Politécnica de Madrid Ethics Committee (December 21, 2015). All
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