

1 **Bone metabolic responses to low energy availability achieved by diet or exercise in active**  
2 **eumenorrheic women**

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24 **Abstract**

25 **Purpose:** We aimed to explore the effects of low energy availability (EA)[15 kcal·kg lean body mass  
26 (LBM)<sup>-1</sup>·d<sup>-1</sup>] achieved by diet or exercise on bone turnover markers in active, eumenorrheic women.

27 **Methods:** By using a crossover design, ten eumenorrheic women (VO<sub>2 peak</sub>: 48.1±3.3 ml·kg<sup>-1</sup>·min<sup>-1</sup>)  
28 completed all three, 3-day conditions in a randomised order: controlled EA (CON; 45 kcal·kgLBM<sup>-1</sup>·d<sup>-1</sup>)  
29 <sup>1</sup>), low EA through dietary energy restriction (D-RES; 15 kcal·kgLBM<sup>-1</sup>·d<sup>-1</sup>) and low EA through  
30 increasing exercise energy expenditure (E-RES; 15 kcal·kgLBM<sup>-1</sup>·d<sup>-1</sup>), during the follicular phase of  
31 three menstrual cycles. In CON, D-RES and E-RES, participants consumed diets providing 45, 15 and  
32 45 kcal·kgLBM<sup>-1</sup>·d<sup>-1</sup>. In E-RES only, participants completed supervised running sessions (129±10  
33 min·d<sup>-1</sup>) at 70% of their VO<sub>2 peak</sub> that resulted in an exercise energy expenditure of 30 kcal·kg LBM<sup>-1</sup>·d<sup>-1</sup>  
34 <sup>1</sup>. Blood samples were collected at baseline (BASE) and at the end of the 3-day period (D6) and analysed  
35 for bone turnover markers (β-CTX and PINP), markers of calcium metabolism (PTH, albumin-adjusted  
36 Ca, Mg and PO<sub>4</sub>) and hormones (IGF-1, T<sub>3</sub>, insulin, leptin and 17β-oestradiol). **Results:** In D-RES,  
37 PINP concentrations at D6 decreased by 17% (BASE: 54.8±12.7 μg·L<sup>-1</sup>, D6: 45.2±9.3 μg·L<sup>-1</sup>, P<0.001,  
38 *d*=0.91) and were lower than D6 concentrations in CON (D6: 52.5±11.9 μg·L<sup>-1</sup>, P=0.001). PINP did  
39 not change significantly in E-RES (BASE: 55.3±14.4 μg·L<sup>-1</sup>, D6: 50.9±15.8 μg·L<sup>-1</sup>, P=0.14). β-CTX  
40 concentrations did not change following D-RES (BASE: 0.48±0.18 μg·L<sup>-1</sup>, D6: 0.55±0.17 μg·L<sup>-1</sup>) or  
41 E-RES (BASE: 0.47±0.24 μg·L<sup>-1</sup>, D6: 0.49±0.18 μg·L<sup>-1</sup>) (condition x time interaction effect, P=0.17).  
42 There were no significant differences in PINP (P=0.25) or β-CTX (P=0.13) responses between D-RES  
43 and E-RES. Both conditions resulted in reductions in IGF-1 (-13% and -23% from BASE in D-RES  
44 and E-RES, both P<0.01) and leptin (-59% and -61% from BASE in D-RES and E-RES, both P<0.001);  
45 T<sub>3</sub> decreased in D-RES only (-15% from BASE, P=0.002) and PO<sub>4</sub> concentrations decreased in E-RES  
46 only (-9%, P=0.03). **Conclusions:** Low EA achieved through dietary energy restriction resulted in a  
47 significant decrease in bone formation but no change in bone resorption, whereas low EA achieved  
48 through exercise did not significantly influence bone metabolism. Both low EA conditions elicited  
49 significant and similar changes in hormone concentrations.

50 **Keywords:** energy availability, dietary energy restriction, exercise energy expenditure, bone  
51 metabolism, active eumenorrheic women

## 52 1. Introduction<sup>1</sup>

53 Active individuals may experience low energy availability (EA) though dietary energy restriction,  
54 exercise energy expenditure or a combination of the two [1]. Low EA has been associated with low  
55 bone mass, impaired bone micro-architecture and increased risk for stress fracture injury [2-5]. These  
56 unfavourable bone outcomes have been highlighted by the Female Athlete Triad [6, 7] and the Relative  
57 Energy Deficiency in Sports (RED-S) model [8]. Short-term studies are important for providing insight  
58 into the time course over which bone metabolic changes occur, when periods of low EA are initiated  
59 [9-11]. We have previously shown that five days of low EA, at 15 kcal·kgLBM<sup>-1</sup>·d<sup>-1</sup>, achieved through  
60 dietary energy restriction and exercise energy expenditure resulted in decreased bone formation and  
61 increased bone resorption in active, eumenorrheic women, but not men [9]. The individual contribution  
62 of exercise and diet on these responses, however, is unknown.

63 To date, short-term studies (< 7 days) that have compared the effects of low EA by different modalities  
64 (*i.e.*, diet *vs.* exercise or diet *vs.* diet plus exercise) are lacking, and findings from long-term  
65 interventional studies on bone parameters are equivocal. Previous research has shown no skeletal  
66 benefits from exercise in weight loss programmes [12, 13], maintenance of bone mass with either  
67 dietary energy restriction and/or exercise-induced energy restriction [14, 15] or amelioration and  
68 prevention of weight loss-associated bone loss with the addition of exercise [16-19]. Notably, these  
69 studies have been conducted in middle-aged [13, 16, 17] or elderly [12, 18, 19] overweight and obese  
70 populations, but no previous study has been performed in active, eumenorrheic women. Most trials have  
71 compared diet to diet plus exercise [12, 13, 18], but few have compared diet to exercise alone [16, 17].  
72 and have utilised exercise protocols suitable for obese/overweight (*i.e.*, exercise of lower intensity or

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### <sup>1</sup> Abbreviations

ACa, Albumin-adjusted calcium; ANOVA, Analysis of variance; AUC, Area under the curve; BASE, Baseline; BMD, Bone mineral density; BMI, Body mass index; CON, Controlled energy availability trial;  $\beta$ -CTX,  $\beta$ -carboxyl-terminal cross-linked telopeptide of type I collagen; D, Day; D-RES: Low energy availability trial though diet; CV, Coefficient of variation; DXA, Dual energy X-ray absorptiometry; EA, Energy availability; E-RES, Low EA trial though exercise; ECLIA, Electrochemiluminescence immunoassay; EDTA, Ethylenediaminetetraacetic acid;; ELISA, Enzyme-linked immunosorbent assay; IGF-1, Insulin-like growth factor 1; IPAQ, International physical activity questionnaire; LBM, Lean body mass; MET, Metabolic equivalent; Mg, Magnesium; P1CP, Carboxyl-terminal propeptide of procollagen type 1; P1NP, Amino-terminal propeptide of procollagen type 1; PO<sub>4</sub>, Phosphate; PTH, Parathyroid hormone; RED-S, Relative energy deficiency in sport, SD, Standard deviation; T<sub>3</sub>, Triiodothyronine; VO<sub>2</sub> peak, Peak aerobic capacity.

73 exercise modes offering no or limited osteogenic stimulus, such as walking and cycling), rather than  
74 active individuals. Given that active individuals practise periods of intense training resulting in high  
75 exercise energy expenditure, which is not accompanied by an increase in dietary energy intake and/or  
76 severely restrict their dietary energy intake during non-training days or the off-season [1, 20], it would  
77 be valuable to explore the effects of low EA, attained by diet or exercise, on bone metabolic responses  
78 in this population.

79 Low EA has been associated with changes in metabolic and reproductive hormones [4, 5, 9, 10], which  
80 may depend on the way in which low EA is achieved [21-23]. Acute energy deficit (approximately 800-  
81 1200 kcal·d<sup>-1</sup>) achieved via dietary energy restriction results in decreased peptide YY and increased  
82 ghrelin concentrations, but no compensatory alterations occur after exercise-induced energy deficit [21,  
83 22]. In contrast, leptin and insulin appear to be similarly reduced in response to low EA achieved  
84 through dietary restriction alone or combined with exercise [24]. Further evidence from studies on  
85 anorexia nervosa and functional hypothalamic amenorrhoea suggest that low EA, regardless of origin,  
86 results in oestrogen deficiency with negative consequences for bone health [23]. A systematic approach  
87 to simultaneously determine changes in bone metabolism, metabolic and reproductive hormone  
88 responses to diet- and exercise-induced low EA in women with normal bone health and reproductive  
89 function is lacking.

90 The aim of this study was to examine and compare the effects of low EA, at 15 kcal·kgLBM<sup>-1</sup>·d<sup>-1</sup>,  
91 achieved by either dietary energy restriction or exercise energy expenditure on bone turnover markers  
92 in active, eumenorrhoeic women.

93 **2. Methods**

94 **2.1. Participants**

95 Ten eumenorrhic women (Table 1.) provided written informed consent to take part in the study. The  
96 study was approved by the Nottingham Trent University Human Research Ethics Committee and the  
97 East Midlands NHS Research Ethics Committee (14/EM/1156) and was conducted in accordance with  
98 the Declaration of Helsinki. Inclusion criteria were 1) age: 18-40 years, 2) Caucasian, 3) self-reported  
99 regular and frequent menstrual cycles (menstrual cycle interval between 24 and 35 days), 4) currently  
100 injury free, 5) participation in moderate and vigorous exercise for  $\geq 3$  hours $\cdot$ week<sup>-1</sup> and 6) BMI between  
101 18.5 and 30 kg $\cdot$ m<sup>-2</sup>. Exclusion criteria were 1) use of medication or suffering from any condition known  
102 to affect bone metabolism, 2) bone fracture within the previous year, 3) current smokers, 4)  
103 breastfeeding, 5) pregnancy, 6) use of any type of hormonal contraception within the past six months  
104 and 7) self-reported short (<24 days), long (>35 days) or irregular menstrual cycles. These criteria were  
105 confirmed verbally and in writing by a health screen with the experimenters, menstrual cycle  
106 questionnaire and the short-form version of International Physical Activity Questionnaire (IPAQ) [25].

107 **Table 1.** Baseline participant characteristics (n=10). (Size: 1column)

<b>Demographics</b>	
Age (y)	24±3
Height (m)	1.66±0.05
Body mass (kg)	61.1±7.0
BMI (kg·m <sup>-2</sup> )	22.3±2.4
<b>Body composition</b>	
Body fat (%)	29.3±5.1
LBM (kg)	41.3±4.1
Fat-free mass (kg)	44.3±4.3
BMD T-score	1.10±0.84
<b>Menstual cycle characteristics</b>	
Length of menstrual cycle (d)	28.5±3.7
Length of flow (d)	4.9±0.9
<b>Training characteristics</b>	
VO <sub>2 peak</sub> (ml·kg <sup>-1</sup> ·min <sup>-1</sup> )	48.1±3.3
VO <sub>2 peak</sub> (ml·kgLBM <sup>-1</sup> ·min <sup>-1</sup> )	70.9±2.8
Physical activity (MET·min·week <sup>-1</sup> )	4634±2382
<b>Dietary and energy expenditure characteristics</b>	
<sup>1</sup> Habitual dietary energy intake (kcal·d <sup>-1</sup> )	2092±262
<sup>1</sup> Lifestyle energy expenditure (kcal·d <sup>-1</sup> )	422±123
<sup>1</sup> Habitual EA (kcal·kgLBM·d <sup>-1</sup> )	39.0±5.6

108 Values are expressed as means±1SD.

109 <sup>1</sup>Analysis performed in 8 participants with complete dietary and energy expenditure data.

110 BMI: Body Mass Index; LBM: Lean Body Mass; BMD: Bone Mineral Density; VO<sub>2 peak</sub>: Peak Oxygen Capacity;

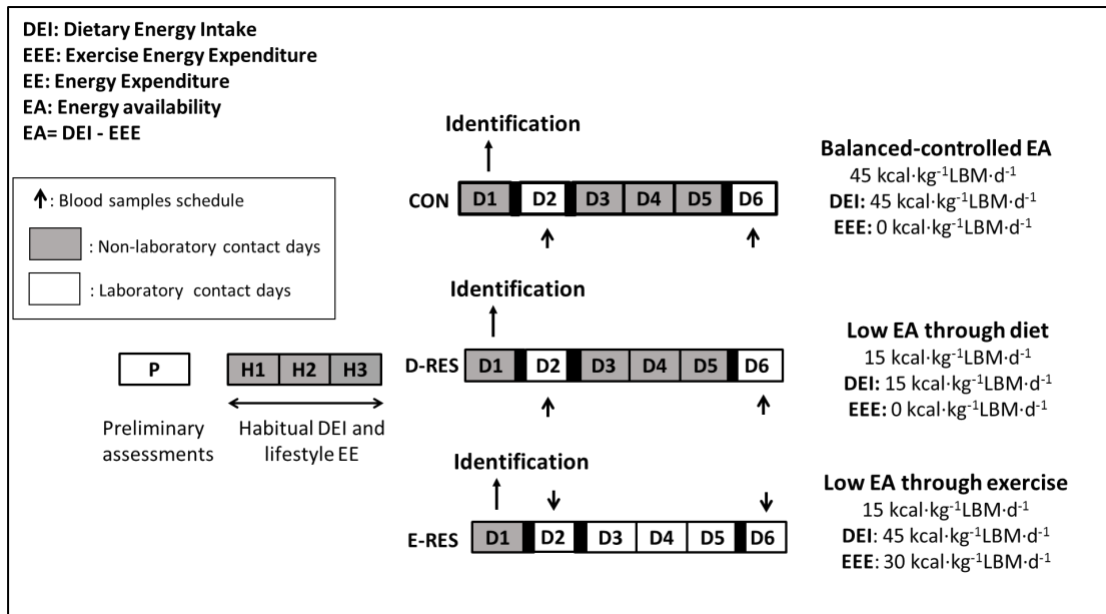
111 MET: Metabolic Equivalents; d: days; EA: Energy Availability.

## 2.2. Experimental design

The study utilised a randomised (Latin square design), crossover design. Participants completed all three experimental conditions; energy-balanced, controlled EA (CON), low EA through diet (D-RES) and low EA through exercise (E-RES) (Figure 1.). Participants attended an initial preliminary visit (P) to establish inclusion criteria, take baseline measurements and determine their fitness level. They also completed a 3-day habitual dietary intake assessment (H1-H3). Participants notified the researchers at the onset of menstruation (D1), which indicated the first day of the experimental study. On the next morning (D2), a blood sample was collected and used as the baseline (BASE) sample prior to each experimental condition. The following 3 days of the protocol (D3-D5) were the experimental condition days. Over D3-D5, participants undertook CON, E-RES and D-RES. On D6, participants had their body mass measured and had a follow-up blood sample (Figure 1.). Due to scheduling constraints, such as the availability of participants or the laboratory, it should be acknowledged that D2 may reflect the second or third day of participants' menstrual cycle, with subsequent small deviations ( $\pm 1$  day) in the main experimental period (D3-D5). For consistency, we will refer to D2 as BASE, D3-D5 as the main experimental period and D6 as the follow-up.

The controlled EA was set at  $45 \text{ kcal}\cdot\text{kgLBM}^{-1}\cdot\text{d}^{-1}$  and achieved by dietary energy intake providing  $45 \text{ kcal}\cdot\text{kgLBM}^{-1}\cdot\text{d}^{-1}$  without exercise. Both low EA conditions (E-RES and D-RES) were administered as  $15 \text{ kcal}\cdot\text{kgLBM}^{-1}\cdot\text{d}^{-1}$ , with this being achieved by dietary energy restriction in D-RES and by exercise energy expenditure in E-RES. In D-RES, participants refrained from exercise and in E-RES, participants completed daily exercise sessions (duration:  $129\pm 10$  min per day) at an exercise intensity of 70% of their peak aerobic capacity ( $\text{VO}_{2 \text{ peak}}$ ) that resulted in an exercise energy expenditure of  $30 \text{ kcal}\cdot\text{kgLBM}^{-1}\cdot\text{d}^{-1}$ , with dietary energy intake at  $45 \text{ kcal}\cdot\text{kgLBM}^{-1}\cdot\text{d}^{-1}$  (Figure 1.). The onset of conditions was typically separated by approximately 28 days, due to each session being initiated in the early follicular phase of the menstrual cycle.





139

140 **Figure 1.** Overview of the study design. Participants completed all three experimental conditions; CON  
 141 controlled EA (CON), low EA through diet (D-RES) and low EA through exercise (E-RES).  
 142 Preliminary day (P) was performed before the first condition was performed. D1: Day 1 of menstruation  
 143 and identification for experimental protocol initiation. D2: Baseline Testing, D3-D5: Condition Days  
 144 and D6: Follow-up Testing. (Size: 2 columns)

## 145 **2.3. Experimental procedures**

### 146 **2.3.1. Preliminary assessment**

147 Participants were weighed wearing tights and t-shirts without shoes on a weighing scale (Seca 875, UK),  
148 height was obtained barefoot using a stadiometer (Seca 217, UK) and BMI was calculated as body mass  
149 (kg) divided by the height squared ( $\text{m}^2$ ). Whole body Dual-energy X-Ray Absorptiometry (DXA; GE  
150 Lunar Prodigy Healthcare) scans were performed to assess body composition (LBM, fat mass) and  
151 baseline bone mineral density (BMD). All DXA scans were conducted and analysed by the same  
152 operator at Nottingham Trent University according to manufacturer's guidelines. Participants provided  
153 a urine sample prior to the DXA scan to confirm normal hydration status ( $<800 \text{ mOsmol}\cdot\text{kg}^{-1}$ ) via urine-  
154 specific gravity (Osmocheck<sup>TM</sup> refractometer, 2595-ED4, Vitech-Scientific, UK).

155 Participants performed a sub-maximal incremental test and a  $\text{VO}_{2 \text{ peak}}$  test on a motorised treadmill, (HP  
156 Cosmos, Germany) using the protocol of [26], to establish the relationship between running speed and  
157 oxygen consumption during level running. Expired gas samples were continuously collected and  
158 analysed by a breath-to-breath automated gas analysis system (ZAN, nSpire Health, Germany). The  
159 running speed at each stage of the speed lactate incremental test was plotted against oxygen  
160 consumption ( $\text{mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ) to determine the sub-maximal relationship between speed and oxygen  
161 consumption and, in combination with  $\text{VO}_{2 \text{ peak}}$ , was used to estimate the running speed corresponding  
162 to 70%  $\text{VO}_{2 \text{ peak}}$  at 0% gradient for the experimental exercise protocol. All participants were given  
163 accelerometers and food weighing scales (Home Digital Kitchen Scale, UK) to record lifestyle energy  
164 expenditure and habitual dietary energy intake over a 3-day lead in period (H1-H3; Figure 1).

### 165 **2.3.2. Habitual Dietary Energy Intake and Lifestyle Energy Expenditure**

166 Participants weighed and recorded food intake during H1-H3 to provide information about their habitual  
167 dietary energy intake. Dietary analysis (macronutrient composition in g and % of total dietary energy  
168 intake) was performed by using Microdiet<sup>TM</sup> software. Participants wore an accelerometer  
169 (GT3X/GT3XE, Actigraph, Pensacola, FL) during all waking hours, except while bathing, to estimate

170 lifestyle energy expenditure. The equation developed by Freedson et al [27] was used to extract lifestyle  
171 energy expenditure data.

### 172 **2.3.3. Experimental diets**

173 In CON, D-RES and E-RES participants consumed diets providing 45, 15 and 45 kcal·kgLBM<sup>-1</sup>·d<sup>-1</sup>.  
174 The experimental diets consisted of the same commercial food products and had standardised  
175 composition (50% carbohydrates, 20% protein and 30% fat) in all experimental conditions. A registered  
176 dietitian designed menus for CON trial (45 kcal·kgLBM<sup>-1</sup>·d<sup>-1</sup>) for a reference individual with a LBM of  
177 45 kg using Microdiet™ software. For the same reference individual, quantities of all food items in  
178 CON (45 kcal·kgLBM<sup>-1</sup>·d<sup>-1</sup>) were divided by three in D-RES (15 kcal·kgLBM<sup>-1</sup>·d<sup>-1</sup>), but were  
179 unchanged in E-RES (45 kcal·kgLBM<sup>-1</sup>·d<sup>-1</sup>). Food quantities in all menus and conditions were  
180 multiplied by a scaling factor to account for differences in LBM compared to the reference individual.  
181 All meals were weighed to the nearest 1 g (Home Digital Kitchen Scale, UK) and were packaged by the  
182 study investigators. Adherence to the diets was verbally confirmed throughout the protocol by asking  
183 the participants whether they consumed the pre-packaged food items in the quantities provided. A  
184 multivitamin, multi-mineral supplement (A - Z Tablets, Boots, Nottingham, UK-nutritional information  
185 of this product is available online: <http://www.boots.com/boots-a-z-90-tablets-10149653>) was supplied  
186 during D-RES to provide adequate micronutrient intake.

### 187 **2.3.4. Exercise energy expenditure**

188 In E-RES only, participants completed exercise sessions that resulted in an exercise energy expenditure  
189 of 30 kcal·kgLBM<sup>-1</sup>·d<sup>-1</sup>. Participants ran on a flat treadmill while being continuously supervised.  
190 Exercise intensity was controlled by setting the treadmill speed to achieve 70% of VO<sub>2 peak</sub> for each  
191 participant and exercise was administered in 15-minute bouts, with 5-minute rest periods between bouts.  
192 Small adjustments in running speed were made throughout the running protocol to maintain the exercise  
193 intensity at 70% of VO<sub>2 peak</sub>. To increase compliance, the total duration of the exercise per day was split  
194 into two sessions of equal duration. Expired gases were continuously collected and analysed using a  
195 breath-by-breath analyser (ZAN 600, nSpire Health, Germany). The required duration of exercise was

196 determined using the oxygen uptake values and respiratory exchange ratio during the first exercise  
197 session (D3) and gas analysis was not performed during the remainder of the exercise sessions (D3  
198 afternoon-D5) to enhance compliance. Outside of the prescribed exercise, participants were instructed  
199 to refrain from exercise and perform only sedentary activities.

#### 200 **2.4. Storage and analyses of blood samples**

201 Blood samples were obtained at the same time of day for each participant and between 07.30-08.15 h  
202 after an overnight fast (from 20:00 h the previous evening) on D2 (BASE) and D6. For plasma  
203 [ethylenediaminetetraacetic acid (EDTA) tubes, SARSTED, Nümbrecht, Germany], samples were  
204 centrifuged immediately at 1509 x g for 10 min at 4°C. Venous blood was dispensed into serum tubes  
205 and allowed to clot at room temperature for 30 min before being centrifuged under the same conditions.  
206 Resultant plasma and serum were aliquoted into Eppendorf tubes and stored at -80°C.  $\beta$ -carboxyl-  
207 terminal cross-linked telopeptide of type I collagen ( $\beta$ -CTX), amino-terminal propeptide of type 1  
208 procollagen (PINP), parathyroid hormone (PTH) and IGF-1 were analysed in EDTA plasma and leptin,  
209 insulin,  $T_3$ ,  $17\beta$ -oestradiol, albumin, calcium (Ca), magnesium (Mg) and phosphate ( $PO_4$ ) in serum.

#### 210 **2.5. Biochemical analysis**

211  $\beta$ -CTX, PINP, PTH,  $T_3$  and  $17\beta$ -oestradiol were measured using electro-chemiluminescence  
212 immunoassay (ECLIA) (Roche Diagnostics, Burgess Hill, UK) on a Cobas e601 analyser. Inter-assay  
213 coefficient of variation (CV) for  $\beta$ -CTX was <3% between 0.2 and 1.5  $\mu\text{g}\cdot\text{L}^{-1}$  with sensitivity of 0.01  
214  $\mu\text{g}\cdot\text{L}^{-1}$ . PINP inter-assay CV was <3% between 20-600  $\mu\text{g}\cdot\text{L}^{-1}$  with a sensitivity of 8  $\mu\text{g}\cdot\text{L}^{-1}$ . PTH inter-  
215 assay CV was <4% between 1-30  $\text{pmol}\cdot\text{L}^{-1}$  with a sensitivity of 0.8  $\text{pmol}\cdot\text{L}^{-1}$ . Sclerostin was measured  
216 using an enzyme-linked immunosorbent assay (ELISA) supplied by Biomedica GmbH (Vienna Austria)  
217 with a sensitivity of 2.6  $\text{pmol}\cdot\text{L}^{-1}$ , which was established from precision profiles (22% CV of duplicates)  
218 and had a CV of <15% across the range 25-95  $\text{pmol}\cdot\text{L}^{-1}$ .  $T_3$  inter-assay CV was <1% between 2.0-3.1  
219  $\text{nmol}\cdot\text{L}^{-1}$  with a detection limit of 0.3  $\text{nmol}\cdot\text{L}^{-1}$ . The inter-assay CV for  $17\beta$ -oestradiol was <3% between  
220 214.3-2156.7  $\text{pmol}\cdot\text{L}^{-1}$  with a detection limit of 18.4  $\text{pmol}\cdot\text{L}^{-1}$ . Leptin was measured using ELISA  
221 (Biovendor, Czech Republic) and had an inter-assay CV of <7% across the range 1-50  $\mu\text{g}\cdot\text{L}^{-1}$  and a

222 sensitivity of 0.2  $\mu\text{g}\cdot\text{L}^{-1}$ . IGF-1 was measured using ELISA (Immunodiagnostic Systems Ltd, Boldon,  
223 UK) and had an inter-assay CV of <2.2% between 24.0-306.2  $\text{ng}\cdot\text{mL}^{-1}$  and a sensitivity of 4.4  $\text{ng}\cdot\text{mL}^{-1}$ .  
224 Insulin was measured using ECLIA (Roche Diagnostics, Burgess Hill, UK), inter-assay CV was <6.1%  
225 across the range 44-505  $\text{pmol}\cdot\text{L}^{-1}$  and sensitivity was 1.8  $\text{pmol}\cdot\text{L}^{-1}$ . Ca, albumin and  $\text{PO}_4$  were measured  
226 using standard commercial assays supplied by Roche Diagnostics performed on the Roche COBAS  
227 c501. The range of measurement in serum was 0.05-5.00  $\text{mmol}\cdot\text{L}^{-1}$  for Ca, 10-70  $\text{g}\cdot\text{L}^{-1}$  for albumin and  
228 0.10-6.46  $\text{mmol}\cdot\text{L}^{-1}$  for  $\text{PO}_4$ . Fluctuations in protein concentrations, especially albumin, may cause total  
229 Ca concentrations to change independently of the ionised calcium concentration, as such Ca  
230 concentrations were 'corrected' to give an albumin-adjusted calcium (ACa) value using the following  
231 equation:  $(-0.8 *([\text{Albumin}] - 4)) + [\text{Total Ca}]$ . Mg was measured using a commercial assay supplied  
232 by Roche Diagnostics and analysed on a COBAS c501. The inter-assay CV was 0.9% across the range  
233 0.1-2.0  $\text{mmol}\cdot\text{L}^{-1}$  and the sensitivity was 0.05  $\text{mmol}\cdot\text{L}^{-1}$ .

## 234 **2.6. Statistical analysis**

235 Based on the results of our previous low EA study [9] in active women, in which low EA was achieved  
236 by a combination of dietary restriction and exercise energy expenditure, the present study was powered  
237 to detect a change in P1NP (pre:  $70.1 \pm 15.1 \text{ mg}\cdot\text{L}^{-1}$ ; follow-up after 3-days:  $60.1 \pm 11.6 \text{ mg}\cdot\text{L}^{-1}$ ,  
238  $P < 0.0001$ ) due to low EA achieved by dietary energy restriction or exercise energy expenditure. An a  
239 priori power calculation determined that 8 women were required to achieve 80% power at  $P < 0.05$ .  
240 Statistical analysis was carried out using Statistica 13.0 (Statsoft, USA). All data were checked for  
241 normality according to the Shapiro-Wilk test and logarithmic transformations were employed for non-  
242 normally distributed data prior to statistical analyses. Baseline biochemistry and body mass prior to  
243 each experimental condition were compared with one-way repeated measures ANOVA, to assess for  
244 differences at baseline. A two-way, repeated measures ANOVA was performed to assess differences  
245 between the experimental conditions (CON, D-RES and E-RES) over time (BASE, D6) for body mass,  
246 bone turnover markers, markers of calcium metabolism, metabolic and reproductive hormones.  
247 Significant main or interaction effects were followed by Tukey's *post-hoc* analysis. Data are presented

248 as mean $\pm$ 1SD and effect sizes (Cohen's *d*; small  $\geq 0.20$ , medium  $\geq 0.50$ , large  $\geq 0.80$ ) [28] are reported.

249 Statistical significance was accepted at the 5% level.

250 In addition to the statistical analysis performed on the whole data set, the individual responses of the  
251 bone turnover markers to D-RES and E-RES were also explored. To be considered a responder,  $\beta$ -CTX  
252 concentrations at D6 in D-RES or E-RES were  $>$ BASE (100%),  $>$   $\beta$ -CTX concentrations at D6 in CON  
253 together with a difference  $>3\%$  to account for CV of  $\beta$ -CTX assay. For P1NP, responders were  
254 identified if P1NP concentrations at D6 in D-RES or E-RES were  $<$ BASE (100%),  $<$ P1NP  
255 concentrations at D6 in CON together with a difference  $>3\%$  to account for CV for P1NP assay.

256 **3. Results**

257 **3.1. Baseline biochemistry and body mass**

258 There were no significant differences in any bone turnover marker, marker of calcium metabolism,  
259 metabolic or reproductive hormone between CON, D-RES and E-RES at BASE (all P-values 0.25-0.90)  
260 (Table 2.). There were no differences in body mass prior to CON, D-RES and E-RES (CON: 60.9±7.0  
261 kg, D-RES: 61.5±7.0 kg, E-RES: 61.1±6.3 kg; P=0.48).

262 **3.2. Body mass**

263 Body mass was reduced at D6 compared to BASE (main effect of time, P<0.001), but did not differ  
264 between conditions (main effect of condition, P=0.82). A significant condition x time interaction effect  
265 (P<0.001) was shown for body mass. Post-hoc analysis showed a trend towards a reduction in body  
266 mass in CON (BASE: 60.9±7.0kg, D6:60.3±6.7 kg, P=0.053, *d*=0.1). Body mass significantly  
267 decreased from BASE in D-RES (BASE: 61.4±6.8 kg, D6: 59.6±6.5 kg, P<0.001) and E-RES (BASE:  
268 61.1±6.3 kg, D6: 60.1±6.0 kg, P<0.001). Body mass at D6 in D-RES was also lower than body mass in  
269 CON (P<0.001) at the same time point.

270 **3.3. Bone turnover markers**

271 Mean  $\beta$ -CTX concentrations were increased at D6 compared to BASE (main effect of time, P=0.044).  
272 No difference was shown for  $\beta$ -CTX concentrations between CON, D-RES and E-RES (main effect of  
273 condition, P=0.13) at any time point (condition x time interaction effect, P=0.17) (Table 2.).

274 Mean P1NP concentrations were decreased at D6 compared to BASE (main effect of time, P<0.001),  
275 but did not differ across conditions (main effect of condition, P=0.25). The condition x time interaction  
276 effect approached significance (P=0.052) (Figure 2., Table 2.). Post-hoc analysis showed that P1NP  
277 concentrations at D6 decreased by 17% from BASE in D-RES (P<0.001, *d*=0.91) and were lower than  
278 P1NP concentrations in CON at the same time point (P<0.001; *d*=0.71). In E-RES, P1NP concentrations

279 at D6 were not significantly different from BASE (-8% from BASE,  $P=0.14$ ,  $d=0.30$ ) or from  
280 concentration at D6 in D-RES ( $P=0.10$ ,  $d=0.43$ ) (Table 2.).

### 281 **3.4. Markers of calcium metabolism**

282 Mean PTH concentrations decreased with time (D6<BASE; main effect of time,  $P=0.02$ ). PTH  
283 responses were not different between experimental conditions, as indicated by a non-significant main  
284 effect of time ( $P=0.21$ ) and no condition x time interaction effect ( $P=0.90$ ) (Table 2.).

285 Mean ACa, Mg and  $PO_4$  concentrations did not change over time (all P values 0.22-0.51). ACa and Mg  
286 concentrations did not differ between CON, D-RES and E-RES at BASE or D6 (condition x time  
287 interaction effect, both P values 0.10-0.89).  $PO_4$  concentrations decreased by 9% at D6 from BASE in  
288 E-RES trial only ( $P=0.03$ ,  $d=1.0$ ) (Table 2.).

### 289 **3.5. Metabolic and reproductive hormones**

290 Mean IGF-1 concentrations at D6 decreased from BASE (main effect of time,  $P=0.01$ ). Mean IGF-1  
291 concentrations were lower in D-RES compared to CON (main effect of condition,  $P=0.03$ ). IGF-1  
292 concentrations at D6 in D-RES and E-RES decreased by 13% ( $P=0.009$ ,  $d=0.76$ ) and 23% ( $P<0.001$ ,  
293  $d=0.97$ ) from BASE and were both significantly lower than IGF-1 concentrations at D6 in CON  
294 ( $P<0.001$ ,  $d>1.0$ ) (Table 2.). IGF-1 concentrations at D6 were not significantly different between D-  
295 RES and E-RES ( $P=0.99$ ,  $d=0.83$ ).

296 Mean  $T_3$  concentrations at D6 decreased from BASE (main effect of time,  $P<0.01$ ).  $T_3$  concentrations  
297 at D6 in D-RES were decreased by 15% from BASE ( $P=0.002$ ,  $d>1.0$ ) and were lower than the  
298 concentrations in CON at the same timepoint ( $P=0.02$ ,  $d=0.88$ ) (Table 2.). In E-RES,  $T_3$  concentrations  
299 at D6 were not significantly different from BASE ( $P=0.21$ )

300 Mean insulin concentrations remained unchanged over time (main effect of time,  $P=0.14$ ) and did not  
301 differ by condition at any time point, as suggested by a non-significant main effect of condition ( $P=0.44$ )  
302 or any condition x time interaction effect ( $P=0.07$ ) (Table 2.).



303 Mean leptin concentrations decreased with time (D6<BASE; P<0.001). Overall, leptin concentrations  
304 were different across conditions (main effect of condition, P<0.001; D-RES<CON, P=0.006 and E-RES  
305 <CON, P=0.02). The condition influenced the response over time for leptin concentration (condition x  
306 time interaction effect, P<0.001). Post-hoc analysis showed that leptin concentrations at D6 in CON,  
307 D-RES and E-RES were significantly lower by 30% (P=0.04,  $d=0.72$ ), 59% (P<0.001,  $d>1.0$ ) and 61%  
308 (P<0.001,  $d>1.0$ ) from BASE prior to each experimental condition. Concentrations at D6 in D-RES  
309 (P<0.001,  $d=0.98$ ) and E-RES (P<0.001,  $d=0.80$ ) were also lower than those at D6 in CON (Table 2.).

310 Mean  $17\beta$ -oestradiol concentrations increased over time (main effect of time, P=0.002) which is in line  
311 with the progression of the menstrual cycle. All participants had  $17\beta$ -oestradiol that indicated early or  
312 mid-follicular phase at the end of the protocol (D6, Minimum: 88.1 pmol·L<sup>-1</sup> and Maximum: 293.5  
313 pmol·L<sup>-1</sup>), in accordance with data produced by Stricker et al [29].  $17\beta$ -oestradiol concentrations were  
314 not different between CON, D-RES and E-RES (main effect of condition, P=0.47) over time (condition  
315 x time interaction, P=0.30) (Table 2.).

### 316 **3.6. Individual analysis**

317 Individual responses for  $\beta$ -CTX and P1NP, as well as altered bone metabolism due to increased  $\beta$ -CTX,  
318 decreased P1NP or both, in D-RES and E-RES trials are presented in Figure 1.

319 **Table 2.** Bone turnover markers, markers of calcium metabolism, metabolic and reproductive hormones  
 320 in CON, D-RES and E-RES trials (n=10). Values at D2 were used as BASE prior to each experimental  
 321 condition. (Size: 2 columns)

	CON		D-RES		E-RES	
	BASE	D6	BASE	D6	BASE	D6
<b>Bone turnover markers</b>						
$\beta$ -CTX ( $\mu\text{g}\cdot\text{L}^{-1}$ )	0.50 $\pm$ 0.19	0.51 $\pm$ 0.18	0.48 $\pm$ 0.18	0.55 $\pm$ 0.17	0.47 $\pm$ 0.24	0.49 $\pm$ 0.18
P1NP ( $\mu\text{g}\cdot\text{L}^{-1}$ )	56.7 $\pm$ 16.9	52.5 $\pm$ 11.9	54.8 $\pm$ 12.7	45.2 $\pm$ 9.3 <sup>**</sup>	55.3 $\pm$ 14.4	50.9 $\pm$ 15.8
<b>Markers of calcium metabolism</b>						
PTH (pg·mL <sup>-1</sup> )	4.4 $\pm$ 1.1	3.8 $\pm$ 0.5	4.0 $\pm$ 0.9	3.7 $\pm$ 0.7	4.6 $\pm$ 1.4	4.1 $\pm$ 0.8
ACa (mmol·L <sup>-1</sup> )	2.30 $\pm$ 0.05	2.31 $\pm$ 0.04	2.27 $\pm$ 0.03	2.27 $\pm$ 0.04	2.29 $\pm$ 0.04	2.26 $\pm$ 0.03
Mg (mmol·L <sup>-1</sup> )	0.83 $\pm$ 0.02	0.82 $\pm$ 0.03	0.81 $\pm$ 0.03	0.81 $\pm$ 0.06	0.81 $\pm$ 0.03	0.82 $\pm$ 0.04
PO <sub>4</sub> (mmol·L <sup>-1</sup> )	1.29 $\pm$ 0.12	1.28 $\pm$ 0.12	1.26 $\pm$ 0.14	1.31 $\pm$ 0.08	1.33 $\pm$ 0.15	1.20 $\pm$ 0.10 <sup>*</sup>
<b>Metabolic and reproductive hormones</b>						
IGF-1 (mmol·L <sup>-1</sup> )	205.0 $\pm$ 39.4	225.4 $\pm$ 50.1	202.5 $\pm$ 46.8	173.3 $\pm$ 29.9 <sup>*,**</sup>	220.6 $\pm$ 56.7	169.2 $\pm$ 49.6 <sup>*,**</sup>
T3 (mmol·L <sup>-1</sup> )	1.49 $\pm$ 0.34	1.47 $\pm$ 0.24	1.53 $\pm$ 0.28	1.29 $\pm$ 0.17 <sup>*,**</sup>	1.53 $\pm$ 0.31	1.40 $\pm$ 0.21
Leptin (ng·mL <sup>-1</sup> )	7.6 $\pm$ 3.7	5.1 $\pm$ 3.1 <sup>*</sup>	6.7 $\pm$ 2.2	2.7 $\pm$ 1.9 <sup>*,**</sup>	8.0 $\pm$ 4.9	3.0 $\pm$ 2.4 <sup>*,**</sup>
Insulin (pmol·L <sup>-1</sup> )	31.6 $\pm$ 7.7	36.7 $\pm$ 20.9	33.8 $\pm$ 8.7	28.6 $\pm$ 15.0	36.9 $\pm$ 18.0	20.4 $\pm$ 11.3
17- $\beta$ oestradiol (pmol·L <sup>-1</sup> )	108.9 $\pm$ 33.6	157.3 $\pm$ 53.1	118.9 $\pm$ 29.7	157.9 $\pm$ 62.9	148.3 $\pm$ 92.9	167.0 $\pm$ 72.1

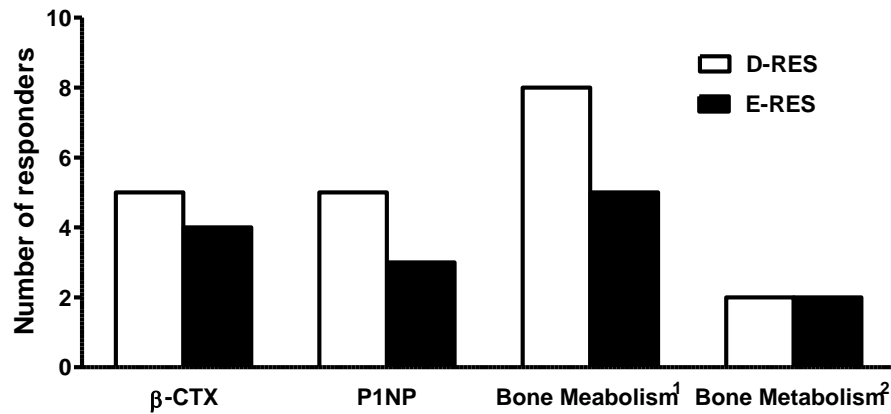
322 Values are expressed as means $\pm$ 1SD.

323 <sup>\*</sup>denotes a significant difference from BASE in the same condition (P<0.05).

324 <sup>\*\*</sup>denotes a significant difference from CON at the same time point (P<0.05).

325

326  $\beta$ -CTX: C-terminal cross-linked telopeptides of type I collagen; P1NP: Amino-terminal pro-peptides of type I  
 327 procollagen; PTH: Parathyroid hormone; Mg: Magnesium; ACa: Albumin adjusted Calcium; PO<sub>4</sub>: Phosphate; T<sub>3</sub>:  
 328 Triiodothyronine; IGF-1: Insulin-like growth factor 1; GLP-2: Glucagon-like peptide 2; BASE: Baseline; CON:  
 329 Controlled EA trial; D-RES: Low EA trial through diet; E-RES: Low EA trial through exercise.



330

331 **Figure 2.** Number of responders (out of total number of participants) for  $\beta$ -CTX, P1NP and bone  
 332 metabolism in D-RES and E-RES. Bone metabolism<sup>1</sup> refers to altered bone metabolism due to increased  
 333  $\beta$ -CTX, decreased P1NP or both. Bone metabolism<sup>2</sup> refers to altered bone metabolism due to a  
 334 simultaneous increase in  $\beta$ -CTX and decrease in P1NP. This analysis was based on data expressed as %  
 335 BASE for each participant. (Size: 2 columns).

336

337  $\beta$ -CTX: C-terminal cross-linked telopeptide of type I collagen; P1NP: Amino-terminal pro-peptides of type I  
 338 procollagen; BASE: Baseline; AUC: Area under the curve; CON: Controlled EA trial; D-RES: Low EA trial  
 339 through diet; E-RES: Low EA trial through exercise.

#### 340 4. Discussion

341 The effects of low EA on bone health in active individuals have received widespread attention in recent  
342 years due to the potential for stress fracture injury and long-term consequences for the development of  
343 osteoporosis [2, 6-8]. Most experimental studies have focused on low EA achieved by a combination  
344 of diet and exercise [9, 10, 30], whereas much less is known about the impact of low EA achieved by  
345 diet or exercise individually. By using a within participant design, our study findings show that 3-days  
346 of low EA achieved by dietary energy restriction resulted in a reduction in bone formation (P1NP,  
347  $P < 0.001$ ,  $d = 0.91$ ), but had no effects on bone resorption. Low EA achieved by exercise energy  
348 expenditure did not significantly affect  $\beta$ -CTX or PINP, our measured markers of bone resorption and  
349 formation. Both low EAs were accompanied by similar reductions in metabolic hormones, suggesting  
350 that these may precede changes in bone turnover markers.

351 The significant P1NP reduction (-17% from BASE), with no further change in  $\beta$ -CTX, shown in  
352 response to low EA achieved via dietary energy restriction, may reflect the degree of EA reached in this  
353 study and the short duration of low EA exposure (3 days). Ihle and Loucks [10] showed that bone  
354 resorption increased following a 5-day experimental period at the most severe level of low EA at 10  
355  $\text{kcal} \cdot \text{kgLBM}^{-1} \cdot \text{d}^{-1}$ , but it remained unaffected at EA of 20  $\text{kcal} \cdot \text{kgLBM}^{-1} \cdot \text{d}^{-1}$ . The importance of the  
356 level of energy restriction for bone metabolism is also supported by a previous short-term fasting study  
357 (4 days), which resulted in synchronous reductions of bone formation (assessed by osteocalcin and  
358 P1CP-carboxyl-terminal propeptide of procollagen type I) and bone formation assessed by urinary  
359 pyridinoline and deoxypyridinoline [11]. Furthermore, in non-obese adults, 12-month exposure to 25%  
360 restriction of dietary energy intake resulted in greater increases in CTX and tartrate-resistant acid  
361 phosphatase (markers of bone resorption) and bone-specific alkaline phosphatase (marker of bone  
362 formation), but not P1NP [31]. Comparisons between studies are, however, difficult to make, due to  
363 differences in study population, duration and selection of bone turnover markers [10, 11, 32]. We  
364 measured P1NP for bone formation, and  $\beta$ -CTX for bone resorption, which are the reference standard  
365 markers in the published literature [33].

366 In our previous study [9], 5 days of low EA at  $15 \text{ kcal}\cdot\text{kgLBM}^{-1}\cdot\text{d}^{-1}$ , achieved by a combination of  
367 dietary energy restriction and exercise energy expenditure, resulted in significantly reduced bone  
368 formation (P1NP area under the curve (AUC); controlled EA:  $-23.1\pm 34.9 \text{ \%BASE x d}$ , low EA:  $-$   
369  $60.9\pm 31.2 \text{ \%BASE x d}$ ;  $P=0.01$ ) and increased bone resorption ( $\beta$ -CTX AUC; controlled EA:  
370  $16.9\pm 68.1\text{ \%BASE x d}$ , low EA:  $85.7\pm 60.5 \text{ \%BASE x d}$ ,  $P=0.03$ ) compared to the controlled condition  
371 ( $45 \text{ kcal}\cdot\text{kgLBM}^{-1}\cdot\text{d}^{-1}$ ) in active eumenorrheic women. These results also support the notion that the  
372 duration of exposure is an important consideration for the negative effect of low EA on bone turnover  
373 markers, and imply that an experimental period longer than 3 days is required to elicit greater changes  
374 in bone metabolism in response to this level of low EA.

375 Exercise-induced low EA at  $15 \text{ kcal}\cdot\text{kgLBM}^{-1}\cdot\text{d}^{-1}$  did not significantly alter bone formation ( $-8\%$  from  
376 BASE in P1NP) or bone resorption ( $+12\%$  from BASE in  $\beta$ -CTX). Our exercise intervention involved  
377 2-2.5 h of running at a moderate intensity ( $70\% \text{ VO}_2 \text{ peak}$ ) for 3 consecutive days, which is a common  
378 training routine followed by some active populations [34, 35]. Our participants were physically active  
379 and habitually performed moderate and vigorous exercise, they were, however, unaccustomed to such  
380 a prolonged duration of daily running over consecutive days. Some osteogenic effects due to the non-  
381 habitual duration and frequency of mechanical loading on weight bearing sites may have occurred [36]  
382 and counterbalanced local bone loss due to low EA in other skeletal sites (e.g., non-weight bearing  
383 sites). In our study the indirect assessment of bone metabolism (by measuring bone turnover markers  
384 in blood samples), provides insight into systemic, rather than localised, effects of low EA.

385 There were no significant differences between the diet-induced and exercise-induced low EAs. It is  
386 uncertain, if the responses in bone turnover markers shown for diet-induced and exercise-induced low  
387 EA would persist over time or whether we were unable to capture any differences due to the short  
388 duration of our experimental protocol. Analysis of changes in bone turnover markers for each individual  
389 showed that 8 out of 10 and 5 out of 10 participants experienced increased  $\beta$ -CTX, decreased P1NP or  
390 both in the diet- and exercise induced low EA trial; suggesting that a subset of women may adversely  
391 respond to low EA, especially following low EA achieved by dietary energy restriction. Further research

392 should expand upon the present study by comparing the effects of low EA achieved by dietary  
393 restriction, exercise energy expenditure, and a combination of both.

394 Whilst we have no comparable data from short-term studies in active individuals, observational studies  
395 suggest that mechanical loading exerts beneficial effects on the skeleton, which may counteract some  
396 of the unfavourable effects of low EA [37-40]. For instance, dancers with amenorrhea (presumably  
397 energy deficient) have greater BMD at weight bearing sites (*e.g.*, proximal femur, lumbar spine)  
398 compared to girls with anorexia nervosa with similarly low body mass, but both groups experience  
399 comparable bone loss at non-weight bearing skeletal sites [40]. Athletes participating in weight sensitive,  
400 non-weight bearing sports (*e.g.*, cyclists, jockeys) are at a greater risk for developing low bone mass  
401 than those partaking in weight bearing activities (*e.g.*, boxers; gymnasts) [37, 38, 39]. Collectively,  
402 these findings suggest that mechanical loading through exercise may have some bone-sparing effects  
403 under long-term energy deficiency and support the findings of this short-term study showing no change  
404 in P1NP concentrations following low EA achieved via exercise energy expenditure, but a reduction in  
405 this bone formation marker when the same of low EA was achieved through dietary energy restriction.

406 The osteoprotective effects of exercise in weight loss programmes have been also demonstrated in  
407 interventional studies in middle-aged or elderly overweight/obese individuals [16-19, 41]. For example,  
408 Villareal et al. reported that the addition of exercise on a weight loss programme ameliorated diet-  
409 induced weight loss reductions in hip BMD in obese older adults [19]. Parallel positive effects were  
410 seen on fat mass, and muscle mass, strength and function [19, 42]. Although there are a number of  
411 differences between lean, active individuals and overweight/obese, mostly sedentary individuals, and  
412 the characteristics of exercise interventions targeting these groups vary greatly, these changes are  
413 suggestive of cross-talk between muscle, adipose tissue and bone under conditions of low EA. Inclusion  
414 of body composition and muscle function measurements, but also, assessment of factors released by  
415 muscles and adipose tissue with potential osteogenic effects [43, 44] in future research in this area will  
416 provide further evidence on the adipose tissue, muscle and bone interactions in response to exercise-  
417 induced low EA in active individuals.

418 Reductions in IGF-1, leptin,  $T_3$  and insulin, indicative of energy conservation, were shown following  
419 the low EA conditions in the present study, which is in agreement with those of short-term energy  
420 deficiency experiments [9-11, 24]. Specifically, decreases in IGF-1 and leptin were shown  
421 independently of whether low EA was achieved by diet or exercise. Decreases in  $T_3$ , however, occurred  
422 in the diet-induced low EA condition only and insulin decreased following exercise-induced low EA  
423 only. When comparing diet- and exercise-induced low EAs, there were no differences in regulatory  
424 hormone concentrations, which is in line with previous findings in a study in active men using the same  
425 level of low EA ( $15 \text{ kcal}\cdot\text{kg LBM}^{-1}\cdot\text{d}^{-1}$ ) achieved through diet only or combined with exercise [24].

426 Neither low EA condition caused a significant change in  $17\beta$ -oestradiol concentrations. These results  
427 are in line with our  $17\beta$ -oestradiol findings following 5 days at the same level of low EA achieved by  
428 dietary energy restriction combined with exercise in active women [9]. In contrast, a 15% reduction in  
429 24-h mean oestrogen concentrations that occurred in parallel with an increase in bone resorption  
430 [urinary N-terminal telopeptide] was reported following 5 days of low EA, attained through diet and  
431 exercise at  $10 \text{ kcal}\cdot\text{kgLBM}^{-1}\cdot\text{d}^{-1}$ , but not  $20 \text{ kcal}\cdot\text{kgLBM}^{-1}\cdot\text{d}^{-1}$  [10]. The discrepancies between the  
432 studies may in part be due to our less severely reduced EA (15 vs.  $10 \text{ kcal}\cdot\text{kgLBM}^{-1}\cdot\text{d}^{-1}$ ) or blood  
433 sampling schedule (single sample vs. 24-h frequent blood collection) [10]. LH pulsatility and  
434 testosterone concentrations, not determined in the current study, may also be negatively affected in  
435 response to low EA [45, 46]. LH pulsatility was suppressed following 5 days of EAs at 10 and 20  
436  $\text{kcal}\cdot\text{kgLBM}^{-1}\cdot\text{d}^{-1}$ , with these findings suggesting that changes in gonadotrophins secreted by the  
437 anterior pituitary may occur prior to changes in ovarian production of oestrogen in states of energy  
438 deficiency [45]. In this study, we purposefully chose the follicular phase phase of the menstrual cycle  
439 because oestrogen levels are less variable compared to other phases of the menstrual cycle. Furthermore,  
440 the initiation of this phase of the menstrual cycle can be easily identified (i.e., first day of bleeding).  
441 Future studies should explore different phases of the menstrual phase (e.g., ovulation, luteal) or include  
442 women with different menstrual status (i.e., amenorrheic or oral contraceptive users), while measuring  
443 more reproductive hormones (i.e., LH pulsatility and testosterone) in relation to bone-related outcomes.

444 Restriction of bone-related macronutrients (*i.e.*, protein) or micronutrients (*i.e.*, calcium) during periods  
445 of energy restriction may contribute to changes in bone metabolism [47]. Herein the variability in  
446 macronutrient distribution within and between participants was eliminated by feeding our participants  
447 with the same food items and dietary composition. We also provided a multi-mineral, multi-vitamin  
448 supplement in the diet-induced low EA trial only to limit the influence of changes in micronutrient  
449 provision. There was, however, a small reduction in PO<sub>4</sub>, when low EA was achieved by exercise energy  
450 expenditure. Changes in systemic PO<sub>4</sub> can have an impact on PTH secretion, with [48] showing that  
451 alterations in PO<sub>4</sub> precede changes in PTH. That said, PTH did not change in the exercise-induced low  
452 EA condition, maybe due to the small PO<sub>4</sub> changes or because of the short timeframe of the study.

## 453 **5. Conclusions**

454 The decrease in bone formation with low EA achieved by diet, but not by exercise alone, suggests that  
455 efforts to protect bone health should possibly focus on improving diet and not by modulating exercise  
456 levels. Future studies with a longer duration of reduced energy availability and a larger sample size  
457 should confirm these findings and identify the mechanisms that mediate low EA effects on bone  
458 turnover markers, since leptin and IGF-1 responded similarly to both conditions in this study.

## 459 **Conflict of interest**

460 None

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466



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