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

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Abstract

Purpose: We aimed to explore the effects of low energy availability (EA)[15 kcal·kg lean body mass (LBM)$^{-1}$·d^{-1}] achieved by diet or exercise on bone turnover markers in active, eumenorrheic women. **Methods:** By using a crossover design, ten eumenorrheic women ($VO_{2\ peak}$: 48.1±3.3 ml·kg^{-1}·min^{-1}) completed all three, 3-day conditions in a randomised order: controlled EA (CON; 45 kcal·kgLBM^{-1}·d^{-1}), low EA through dietary energy restriction (D-RES; 15 kcal·kgLBM^{-1}·d^{-1}) and low EA through increasing exercise energy expenditure (E-RES; 15 kcal·kgLBM^{-1}·d^{-1}), during the follicular phase of three menstrual cycles. In CON, D-RES and E-RES, participants consumed diets providing 45, 15 and 45 kcal·kgLBM^{-1}·d^{-1}. In E-RES only, participants completed supervised running sessions (129±10 min·d^{-1}) at 70% of their $VO_{2\ peak}$ that resulted in an exercise energy expenditure of 30 kcal·kg LBM^{-1}·d^{-1}. Blood samples were collected at baseline (BASE) and at the end of the 3-day period (D6) and analysed for bone turnover markers (β-CTX and P1NP), markers of calcium metabolism (PTH, albumin-adjusted Ca, Mg and PO_4) and hormones (IGF-1, T_3, insulin, leptin and 17β-oestradiol). **Results:** In D-RES, P1NP concentrations at D6 decreased by 17% (BASE: 54.8±12.7 µg·L^{-1}, D6: 45.2±9.3 µg·L^{-1}, P<0.001, *d*=0.91) and were lower than D6 concentrations in CON (D6: 52.5±11.9 µg·L^{-1}, P=0.001). P1NP did not change significantly in E-RES (BASE: 55.3±14.4 µg·L^{-1}, D6: 50.9±15.8 µg·L^{-1}, P=0.14). β-CTX concentrations did not change following D-RES (BASE: 0.48±0.18 µg·L^{-1}, D6: 0.55±0.17 µg·L^{-1}) or E-RES (BASE: 0.47±0.24 µg·L^{-1}, D6: 0.49±0.18 µg·L^{-1}) (condition x time interaction effect, P=0.17). There were no significant differences in P1NP (P=0.25) or β-CTX (P=0.13) responses between D-RES and E-RES. Both conditions resulted in reductions in IGF-1 (-13% and –23% from BASE in D-RES and E-RES, both P<0.01) and leptin (-59% and –61% from BASE in D-RES and E-RES, both P<0.001); T_3 decreased in D-RES only (-15% from BASE, P=0.002) and PO_4 concentrations decreased in E-RES only (-9%, P=0.03). **Conclusions:** Low EA achieved through dietary energy restriction resulted in a significant decrease in bone formation but no change in bone resorption, whereas low EA achieved through exercise did not significantly influence bone metabolism. Both low EA conditions elicited significant and similar changes in hormone concentrations.

Keywords: energy availability, dietary energy restriciton, exercise energy expenditure, bone metabolism, active eumenorrheic women

1. Introduction[1]

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

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

[1] 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; β-CTX, β-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, Electro-chemiluminescence 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_4, Phosphate; PTH, Parathyroid hormone; RED-S, Relative energy deficiency in sport, SD, Standard deviation; T_3, Triiodothyronine; VO_2 peak, Peak aerobic capacity.

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

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

The aim of this study was to examine and compare the effects of low EA, at 15 $kcal \cdot kgLBM^{-1} \cdot d^{-1}$, achieved by either dietary energy restriction or exercise energy expenditure on bone turnover markers in active, eumenorrheic women.

2. Methods

2.1. Participants

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

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

Demographics	
Age (y)	24±3
Height (m)	1.66±0.05
Body mass (kg)	61.1±7.0
BMI ($kg \cdot m^{-2}$)	22.3±2.4
Body composition	
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
Menstual cycle characteristics	
Length of menstrual cycle (d)	28.5±3.7
Length of flow (d)	4.9±0.9
Training characteristics	
$VO_{2\ peak}$ ($ml \cdot kg^{-1} \cdot min^{-1}$)	48.1±3.3
$VO_{2\ peak}$ ($ml \cdot kgLBM^{-1} \cdot min^{-1}$)	70.9±2.8
Physical activity ($MET\text{-}min \cdot week^{-1}$)	4634±2382
Dietary and energy expenditure characteristics	
[1]Habitual dietary energy intake ($kcal \cdot d^{-1}$)	2092±262
[1]Lifestyle energy expenditure ($kcal \cdot d^{-1}$)	422±123
[1]Habitual EA ($kcal \cdot kgLBM \cdot d^{-1}$)	39.0±5.6

Values are expressed as means±1SD.

[1]Analysis performed in 8 participants with complete dietary and energy expenditure data.

BMI: Body Mass Index; LBM: Lean Body Mass; BMD: Bone Mineral Density: VO_2 peak: Peak Oxygen Capacity; 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 (±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 kcal·kgLBM^{-1}·d^{-1} and achieved by dietary energy intake providing 45 kcal·kgLBM^{-1}·d^{-1} without exercise. Both low EA conditions (E-RES and D-RES) were administered as 15 kcal·kgLBM^{-1}·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±10 min per day) at an exercise intensity of 70% of their peak aerobic capacity ($VO_{2\ peak}$) that resulted in an exercise energy expenditure of 30 kcal·kgLBM^{-1}·d^{-1}, with dietary energy intake at 45 kcal·kgLBM^{-1}·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.

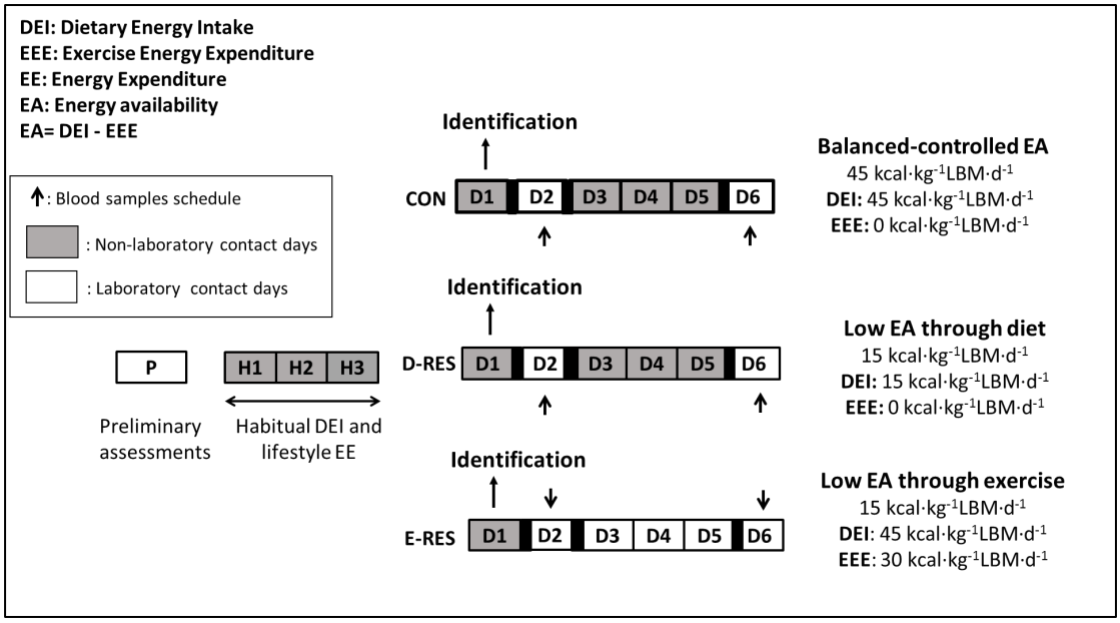


Figure 1. Overview of the study design. Participants completed all three experimental conditions; CON controlled EA (CON), low EA through diet (D-RES) and low EA through exercise (E-RES). Preliminary day (P) was performed before the first condition was performed. D1: Day 1 of menstruation and identification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

2.3. Experimental procedures

2.3.1. Preliminary assessment

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

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

2.3.2. Habitual Dietary Energy Intake and Lifestyle Energy Expenditure

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

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

2.3.3. Experimental diets

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

2.3.4. Exercise energy expenditure

In E-RES only, participants completed exercise sessions that resulted in an exercise energy expenditure of 30 kcal·kgLBM^{-1}·d^{-1}. Participants ran on a flat treadmill while being continuously supervised. Exercise intensity was controlled by setting the treadmill speed to achieve 70% of $VO_{2\ peak}$ for each participant and exercise was administered in 15-minute bouts, with 5-minute rest periods between bouts. Small adjustments in running speed were made throughout the running protocol to maintain the exercise intensity at 70% of $VO_{2\ peak}$. To increase compliance, the total duration of the exercise per day was split into two sessions of equal duration. Expired gases were continuously collected and analysed using a 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 4ºC. 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 -80ºC. β-carboxyl-
207 terminal cross-linked telopeptide of type I collagen (β-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, 17β-oestradiol, albumin, calcium (Ca), magnesium (Mg) and phosphate (PO_4) in serum.

210 **2.5. Biochemical analysis**

211 β-CTX, P1NP, PTH, T_3 and 17β-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 β-CTX was <3% between 0.2 and 1.5 $\mu g \cdot L^{-1}$ with sensitivity of 0.01
214 $\mu g \cdot L^{-1}$. P1NP inter-assay CV was <3% between 20-600 $\mu g \cdot L^{-1}$ with a sensitivity of 8 $\mu g \cdot L^{-1}$. PTH inter-
215 assay CV was <4% between 1-30 $pmol \cdot L^{-1}$ with a sensitivity of 0.8 $pmol \cdot L^{-1}$. Sclerostin was measured
216 using an enzyme-linked immunosorbent assay (ELISA) supplied by Biomedica GmbH (Vienna Austria)
217 with a sensitivity of 2.6 $pmol \cdot L^{-1}$, which was established from precision profiles (22% CV of duplicates)
218 and had a CV of <15% across the range 25-95 $pmol \cdot L^{-1}$. T_3 inter-essay CV was <1% between 2.0-3.1
219 $nmol \cdot L^{-1}$ with a detection limit of 0.3 $nmol \cdot L^{-1}$. The inter-assay CV for 17β-oestradiol was <3% between
220 214.3-2156.7 $pmol \cdot L^{-1}$ with a detection limit of 18.4 $pmol \cdot L^{-1}$. Leptin was measured using ELISA
221 (Biovendor, Czech Republic) and had an inter-assay CV of <7% across the range 1-50 $\mu g \cdot L^{-1}$ and a

sensitivity of 0.2 µg·L^{-1}. IGF-1 was measured using ELISA (Immunodiagnostic Systems Ltd, Boldon, UK) and had an inter-assay CV of <2.2% between 24.0-306.2 ng·mL^{-1} and a sensitivity of 4.4 ng·mL^{-1}. Insulin was measured using ECLIA (Roche Diagnostics, Burgess Hill, UK), inter-assay CV was <6.1% across the range 44-505 pmol·L^{-1} and sensitivity was 1.8 pmol·L^{-1}. Ca, albumin and PO_4 were measured using standard commercial assays supplied by Roche Diagnostics performed on the Roche COBAS c501. The range of measurement in serum was 0.05-5.00 mmol·L^{-1} for Ca, 10-70 g·L^{-1} for albumin and 0.10-6.46 mmol·L^{-1} for PO_4. Fluctuations in protein concentrations, especially albumin, may cause total Ca concentrations to change independently of the ionised calcium concentration, as such Ca concentrations were 'corrected' to give an albumin-adjusted calcium (ACa) value using the following equation: (-0.8 *([Albumin] - 4)) + [Total Ca]. Mg was measured using a commercial assay supplied by Roche Diagnostics and analysed on a COBAS c501. The inter-assay CV was 0.9% across the range 0.1-2.0 mmol·L^{-1} and the sensitivity was 0.05 mmol·L^{-1}.

2.6. Statistical analysis

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

as mean±1SD and effect sizes (Cohen's *d;* small ≥ 0.20, medium ≥ 0.50, large ≥ 0.80) [28] are reported. Statistical significance was accepted at the 5% level.

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

3. Results

3.1. Baseline biochemistry and body mass

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

3.2. Body mass

Body mass was reduced at D6 compared to BASE (main effect of time, P<0.001), but did not differ between conditions (main effect of condition, P=0.82). A significant condition x time interaction effect (P<0.001) was shown for body mass. Post-hoc analysis showed a trend towards a reduction in body mass in CON (BASE: 60.9±7.0kg, D6:60.3±6.7 kg, P=0.053, *d*=0.1). Body mass significantly 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: 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 CON (P<0.001) at the same time point.

3.3. Bone turnover markers

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

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

Mean leptin concentrations decreased with time (D6<BASE; P<0.001). Overall, leptin concentrations were different across conditions (main effect of condition, P<0.001; D-RES<CON, P=0.006 and E-RES <CON, P=0.02). The condition influenced the response over time for leptin concentration (condition x time interaction effect, P<0.001). Post-hoc analysis showed that leptin concentrations at D6 in CON, 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% (P<0.001, *d*>1.0) from BASE prior to each experimental condition. Concentrations at D6 in D-RES (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.).

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

3.6. Individual analysis

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

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

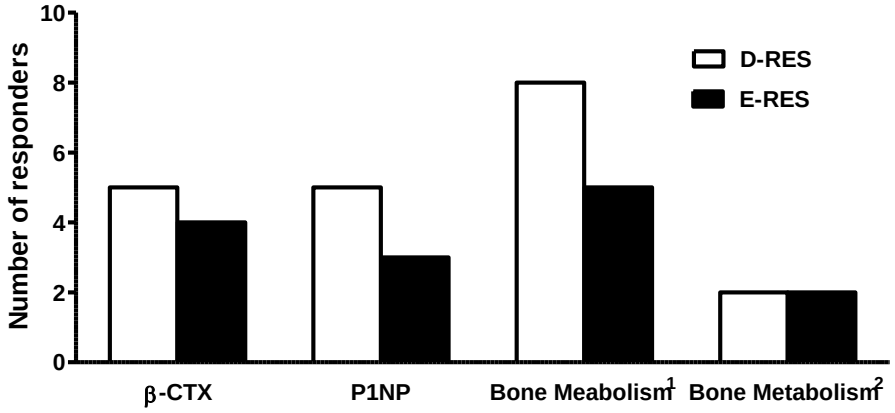
	CON		D-RES		E-RES	
	BASE	**D6**	**BASE**	**D6**	**BASE**	**D6**
Bone turnover markers						
β-CTX ($\mu g \cdot L^{-1}$)	0.50±0.19	0.51±0.18	0.48±0.18	0.55±0.17	0.47±0.24	0.49±0.18
P1NP ($\mu g \cdot L^{-1}$)	56.7±16.9	52.5±11.9	54.8±12.7	45.2±9.3*,**	55.3±14.4	50.9±15.8
Markers of calcium metabolism						
PTH ($pg \cdot mL^{-1}$)	4.4±1.1	3.8± 0.5	4.0±0.9	3.7±0.7	4.6±1.4	4.1±0.8
ACa ($mmol \cdot L^{-1}$)	2.30±0.05	2.31 ±0.04	2.27±0.03	2.27±0.04	2.29±0.04	2.26±0.03
Mg ($mmol \cdot L^{-1}$)	0.83±0.02	0.82±0.03	0.81±0.03	0.81±0.06	0.81±0.03	0.82±0.04
PO_4 ($mmol \cdot L^{-1}$)	1.29±0.12	1.28±0.12	1.26±0.14	1.31±0.08	1.33± 0.15	1.20±0.10*
Metabolic and reproductive hormones						
IGF-1 ($mmol \cdot L^{-1)}$)	205.0± 39.4	225.4± 50.1	202.5± 46.8	173.3± 29.9*,**	220.6± 56.7	169.2± 49.6*,**
T3 ($mmol \cdot L^{-1}$)	1.49±0.34	1.47±0.24	1.53±0.28	1.29± 0.17*,**	1.53±0.31	1.40±0.21
Leptin ($ng \cdot mL^{-1}$)	7.6±3.7	5.1±3.1*	6.7±2.2	2.7±1.9*,**	8.0±4.9	3.0± 2.4*,**
Insulin ($pmol \cdot L^{-1}$)	31.6±7.7	36.7± 20.9	33.8± 8.7	28.6±15.0	36.9±18.0	20.4±11.3
17-β oestradiol ($pmol.L^{-1}$)	108.9± 33.6	157.3± 53.1	118.9± 29.7	157.9± 62.9	148.3± 92.9	167.0± 72.1

Values are expressed as means±1SD.

*denotes a significant difference from BASE in the same condition ($P<0.05$).

**denotes a significant difference from CON at the same time point ($P<0.05$).

β-CTX: C-terminal cross-linked telopeptides of type I collagen; P1NP: Amino-terminal pro-peptides of type 1 procollagen; PTH: Parathyroid hormone; Mg: Magnesium; ACa: Albumin adjusted Calcium; PO_4: Phosphate; T_3: Triiodothyronine; IGF-1: Insulin-like growth factor 1; GLP-2: Glucagon-like peptide 2; BASE: Baseline; CON: 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 *β*-CTX, P1NP and bone
332 metabolism in D-RES and E-RES. Bone metabolism[1] refers to altered bone metabolism due to increased
333 β-CTX, decreased P1NP or both. Bone metabolism[2] refers to altered bone metabolism due to a
334 simultaneous increase in β-CTX and decrease in P1NP. This analysis was based on data expressed as %
335 BASE for each participant. (Size: 2 columns).

336

337 *β*-CTX: C-terminal cross-linked telopeptide of type I collagen; P1NP: Amino-terminal pro-peptides of type 1
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.

4. Discussion

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

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

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

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

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

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

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

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

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

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

Restriction of bone-related macronutrients (*i.e.*, protein) or micronutrients (*i.e.*, calcium) during periods of energy restriction may contribute to changes in bone metabolism [47]. Herein the variability in macronutrient distribution within and between participants was eliminated by feeding our participants with the same food items and dietary composition. We also provided a multi-mineral, multi-vitamin supplement in the diet-induced low EA trial only to limit the influence of changes in micronutrient provision. There was, however, a small reduction in PO_4, when low EA was achieved by exercise energy expenditure. Changes in systemic PO_4 can have an impact on PTH secretion, with [48] showing that alterations in PO_4 precede changes in PTH. That said, PTH did not change in the exercise-induced low EA condition, maybe due to the small PO_4 changes or because of the short timeframe of the study.

5. Conclusions

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

Conflict of interest

None

Acknowledgements

We thank our study participants for their contribution to this investigation.

Funding sources

The study formed part of the programmes of work for MP and DM. MP was supported by a Vice Chancellors Research Student Bursary from Nottingham Trent University.

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