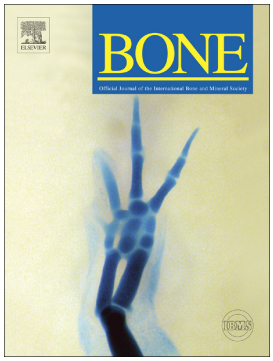


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PII: S8756-3282(17)30313-7
DOI: doi: 10.1016/j.bone.2017.08.019
Reference: BON 11405

To appear in: *Bone*

Received date: 12 January 2017
Revised date: 22 June 2017
Accepted date: 19 August 2017

Please cite this article as: Maria Papageorgiou, Kirsty J. Elliott-Sale, Alan Parsons, Johnathan C.Y. Tang, Julie P. Greeves, William D. Fraser, Craig Sale , Effects of reduced energy availability on bone metabolism in women and men, *Bone* (2017), doi: 10.1016/j.bone.2017.08.019

**Effects of reduced energy availability on bone metabolism in women and men**

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**Abstract**

**Background:** The short-term effects of low energy availability (EA) on bone metabolism in physically active women and men are currently unknown. **Purpose:** We evaluated the effects of low EA on bone turnover markers (BTMs) in a cohort of women and a cohort of men, and compared effects between sexes. **Methods:** These studies were performed using a randomised, counterbalanced, crossover design. Eleven eumenorrheic women and eleven men completed two 5-day protocols of controlled (CON; 45 kcal·kgLBM$^{-1}$·d$^{-1}$) and restricted (RES; 15 kcal·kgLBM$^{-1}$·d$^{-1}$) EAs. Participants ran daily on a treadmill at 70% of their peak aerobic capacity ($VO_2$ peak) resulting in an exercise energy expenditure of 15 kcal·kgLBM$^{-1}$·d$^{-1}$ and consumed diets providing 60 and 30 kcal·kgLBM$^{-1}$·d$^{-1}$. Blood was analysed for BTMs [β-carboxyl-terminal cross-linked telopeptide of type I collagen (β-CTX) and amino-terminal propeptide of type 1 procollagen (P1NP)], markers of calcium metabolism [(parathyroid hormone (PTH), albumin-adjusted calcium (ACa), magnesium (Mg) and phosphate ($PO_4$)] and regulatory hormones [sclerostin, insulin-like growth factor 1 (IGF-1), triiodothyronine ($T_3$), insulin, leptin, glucagon-like- peptide-2 (GLP-2)]. **Results:** In women, β-CTX AUC was significantly higher (P=0.03) and P1NP AUC was significantly lower (P=0.01) in RES compared to CON . In men, neither β-CTX (P=0.46) nor P1NP (P=0.12) AUCs were significantly different between CON and RES. There were no significant differences between sexes for any BTM AUCs (all P values>0.05). Insulin and leptin AUCs were significantly lower following RES in women only (for both P=0.01). There were no differences in any AUCs of regulatory hormones or markers of calcium metabolism between men and women following RES (all P values>0.05). **Conclusions:** When comparing within groups, five days of low EA (15 kcal·kgLBM$^{-1}$·d$^{-1}$) decreased bone formation and increased bone resorption in women, but not in men, and no sex specific differences were detected.

**Keywords:** Female Athlete Triad, Relative Energy Deficiency in Sports, Energy availability, Bone turnover markers, Physically active individuals

## 1. Introduction[1]

Physically active individuals may undertake high training volumes and/or unintentionally or deliberately restrict their dietary energy intake (DEI), practices that can put them at risk for energy deficiency either in isolation or when combined [1]. The concept of energy balance and, more recently, the concept of energy availability (EA), have been used to quantify energy deficiency in physically active individuals. EA is defined as DEI minus exercise energy expenditure (EEE) and is adjusted for muscle mass, whereas energy balance is calculated as total energy expenditure (TEE) minus DEI. EA differs from energy balance in a number of ways; 1. the estimation of TEE required for the determination of energy balance may introduce more sources of errors than the measurement of EEE, which is required for determining EA and 2. the concept of energy balance assumes that bodily systems function normally, however, increases in EEE may suppress bodily functions [1], which means that individuals may be in energy balance but experience low EA. These differences between the two concepts suggest that EA may be advantageous while investigating the effects of energy deficiency in physically active individuals.

Low EA has been associated with menstrual dysfunction and impaired bone health in female athletes, described as the Female Athlete Triad [2, 3]. Cross-sectional studies suggest that similar adverse medical conditions may also be experienced by male athletes, with those participating in weight sensitive sports being at a greater risk [for a review see [4]; [5, 6]]. Based on current evidence, the International Olympic Committee extended the Female Athlete Triad to incorporate aspects of physiological function, athletic performance and health in both sexes [Relative Energy Deficiency

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**[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; Bone turnover ratio; BTM, Bone turnover markers; BT Ratio, CON, Controlled; β-CTX, β-carboxyl-terminal cross-linked telopeptide of type I collagen; D, Day; DEI, Dietary energy intake; CV, Coefficient of variation; DXA, Dual energy X-ray absorptiometry; EA, Energy availability; ECLIA, Electro-chemiluminescence immunoassay; EDTA, Ethylenediaminetetraacetic acid; EEE, Exercise energy expenditure; ELISA, Enzyme-linked immunosorbent assay; IGF-1, Insulin-like growth factor; IPAQ, International physical activity questionnaire; GLP-2, Glucagon-like peptide-2; LBM, Lean body mass; MET, Metabolic equivalent; Mg, Magnesium; NTX, Amino-terminal cross-linked telopeptide of type I collagen; P1NP, Amino-terminal propeptide of procollagen type 1; $PO_4$, Phosphate; PTH, Parathyroid hormone; RED-S, Relative energy deficiency in sport, RES, Restricted; SD, Standard deviation; $T_3$, Triiodothyronine; $VO_{2peak}$, Peak aerobic capacity; Wnt, Wingless.

in Sports (RED-S) models] [7]. High prevalence rates of stress fracture injuries, up to 21% and 49% have been reported in athletes [8] and military recruits [9], with women potentially being at higher risk for this type of injury compared to men [10]. Factors associated with energy deficiency including low EA, low body weight/BMI, disordered eating and rapid weight loss are risk factors for low BMD [5, 11], sustaining a stress fracture injury [12, 13] and experiencing an osteoporotic fracture in later life [14]. Given the potential for reduced bone health of physically active populations both during and after the cessation of their career, it is important to understand the effects of low EA on bone in order to develop effective prevention, early identification and treatment strategies.

Unfavourable bone outcomes may reflect uncoupled bone turnover (decreased bone formation, increased bone resorption or a combination of the two) following low EA. Initial bone metabolic responses to low EA remain unknown. Decreased bone formation, at different levels of low EA [30, 20 and 10 kcal·kg lean body mass $(LBM)^{-1}\cdot d^{-1}$], and increased bone resorption, with severely restricted EA (10 kcal· kg $LBM^{-1}\cdot d^{-1}$) over 5 days, have been shown in sedentary women when compared to balanced EA (45 kcal· kg $LBM^{-1}\cdot d^{-1}$) [15]. Some amenorrheic athletes report EA at ~16 kcal·kg$LBM^{-1}\cdot d^{-1}$ [16], making it important to explore the effects of this level of reduced EA on bone metabolism in physically active women. Controlled EA experiments on the effects of low EA on bone metabolism are lacking in men, with only one study [17] showing that three days of running with 50% dietary energy restriction resulted in reduced bone formation, without an effect on bone resorption. This study was designed on the basis of energy balance rather than EA. As such, well-controlled experimental studies are needed to investigate the effects of low EA on bone metabolism in physically active men, as well as in women.

Physiological adaptations to low EA involve alterations in regulatory hormones, including decreased triiodothyronine ($T_3$), insulin-like growth factor-1 (IGF-1) and leptin [15, 17, 18]. Based on the findings of previous experiments [19, 20], some of these endocrine responses likely differ between men and women at the same level of energy restriction. Low EA also results in the suppression of ovulatory cycles in women, through the inhibition of gonadotropin-releasing hormone, reduced luteinising hormone pulsatility and decreased oestrogen concentrations [18, 21]. Such effects on the hypothalamic pituitary axis are less clear in men due to limited clinical signs and specific analyses of sperm and fertility [22]. Several field studies have shown significant reductions in testosterone concentrations and alterations in markers of

bone metabolism in energy-deficient male athletes [6, 23] although experimental studies are needed to establish cause-effect relationships between EA, reproductive and bone health in men. As both energy regulatory and reproductive hormones affect bone metabolism and bone mineral density (BMD) by acting independently or synergistically [24], different responses to low EA could cause distinct bone metabolic responses that might result in sex differences at given levels of EA.

We conducted two randomised, crossover studies to investigate the effects of short-term low EA, at 15 kcal·kgLBM$^{-1}$·d$^{-1}$ achieved by combined dietary energy restriction and exercise, on bone turnover markers (BTMs) in physically active women and men. A secondary aim was to compare effects between sexes.

## 2. Materials and methods

### 2.1. Experimental Design

A randomised (Standard Latin squares for 2 x 2), counterbalanced, crossover, design was employed. Participants completed two 9-day experimental periods (D 1-9): controlled (CON) balanced (45 kcal·kg$^{-1}$LBM·d$^{-1}$) and restricted (RES) EA (15 kcal·kg$^{-1}$LBM·d$^{-1}$), achieved by manipulating diet and exercise. EA was defined as DEI minus exercise energy expenditure (EEE) adjusted for LBM to consider metabolically active tissue mass and account for individual differences in body composition as recommended by [15]. DEI provided 60 and 30 kcal·kg$^{-1}$LBM·d$^{-1}$ in CON and RES, and participants expended 15 kcal·kg$^{-1}$LBM·d$^{-1}$ while exercising (running) at 70% of their peak aerobic capacity ($VO_2$ peak) (Figure 1). Given the crossover design, experimental conditions were separated by at least one menstrual cycle (~28 d apart) from the start of the one condition to the start of the next condition for female participants and a similar wash-out period was allowed for male participants.

Habitual DEI and lifestyle energy expenditure were recorded for participants between D 1-3, having refrained from all systematic exercise during this period. Over D 4-8, participants undertook CON or RES (Figure 1). Female participants started D 4 (main experimental protocol) of each condition in the early follicular phase (defined as menstruation), as confirmed by 17β-oestradiol measurements on D 5 in both experimental conditions [25]. At D 9, 17β-oestradiol measurements for all participants were within the range for early (1-9 days; median: 149.7 pmol·L-1, 5$^{th}$ percentile: 77.99 pmol·L$^{-1}$, 95$^{th}$ percentile: 266.08 pmol·L$^{-1}$) or late follicular phase (defined as prior to ovulation) (10-14 days: median: 450.5 pmol·L$^{-1}$, 5$^{th}$ percentile 195.43 pmol·L$^{-1}$, 95$^{th}$ percentile: 1146.91 pmol·L$^{-1}$) according to Stricker et al., 2006 [25]. Given the duration of this protocol, some progression towards the late follicular phase was expected. Given that participants started D 4 on the 2$^{nd}$ or 3$^{rd}$ day of bleeding in both conditions, this progression should have been similar between CON and RES (in the absence of potential effects of RES).

The study was approved by the Nottingham Trent University Research Ethics Committee (humans) in accordance with the declaration of Helsinki and written informed consent was obtained from all participants.

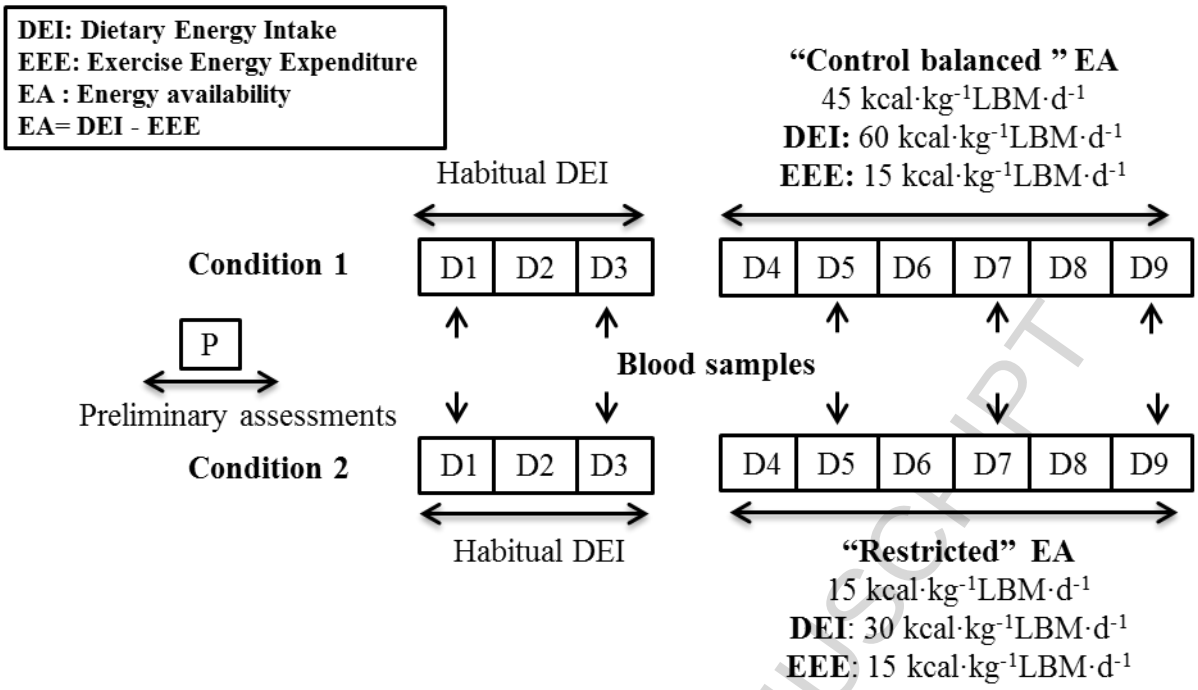


**Figure 1.** Diagram of the study design. P: Preliminary assessments; D 1-D 8: Experimental Days. The gap between D 3 and D 4 denote change into either the CON or RES period; adjoined boxes denote consecutive days; the thick black line denotes when CON/RES EA conditions were stopped. Arrows denote blood sampling. (Size: 2 columns)

### 2.2. Participants

Eleven eumenorrheic women and eleven men (Table 1) volunteered. Participants were included if they were Caucasian, aged 18-35 years, non-smokers, had a body mass index (BMI) between 18.5 and 30 $kg \cdot m^{-2}$, had not sustained a bone fracture within the previous year, were currently injury free, had no history of disordered eating and did not use any medication or suffer from any condition affecting bone metabolism. These criteria were confirmed verbally and by health screen and SCOFF questionnaire, a standard screening tool for eating disorders, for a detailed description see [26]. Self - reported menstrual cycle details were assessed using a questionnaire, which included questions about menstrual frequency and length, bleeding length and previous oral contraceptive pill use. Menstrual cycle length was defined as the number of days from the first day of menstruation to the day before the next onset of menstruation. Eumenorrhea was established if menstruation occurred at regular intervals of 24–35 days [11]. Amenorrheic women (absence of menstruation for a minimum of 3 repeated months), oligomenorrheic women (menstrual cycles of 36–90 days) and women with short

menstrual cycles (menstrual cycles <24 days) were excluded from participation to ensure that existing reproductive disturbances did not affect the findings [11]. Participants regularly performed ≥3 hours of moderate to vigorous physical activity per week and had moderate and high physical activity levels (Table 1), as determined by the International Physical Activity Questionnaire (IPAQ). Participants were familiar with the exercise mode (running) as part of their training routine.

### 2.3. Experimental Procedures

#### 2.3.1. Preliminary Assessments

Height and weight (SECA, UK) were obtained prior to a whole body dual energy X-ray absorptiometry (DXA, Lunar iDXA, GE Healthcare, USA) scan, which was performed to determine body composition in accordance with the relevant regulations. Participants performed an incremental exercise test on a treadmill (HP Cosmos, Germany) to determine their $VO_2$ peak, using previously published methods [27].

#### 2.3.2. Experimental Period

##### 2.3.2.1. Lifestyle energy expenditure

Participants wore an accelerometer (WGT3X-BT; Actigraph, Pensacola, Florida) during all waking hours on D 1-8, except while bathing, to estimate lifestyle energy expenditure. Participants did not wear the acceleropmeters during the exercise protocol on D 4-8.

##### 2.3.2.2. Habitual DEI

Participants weighed and recorded food intake during D 1-3 to provide habitual DEI. Participants were shown how to complete detailed food records and the dietary analysis was performed by a registered dietitian using Microdiet™ software.

#### 2.3.2.3. Experimental Diets

During D 4-8, each participant was given diet plans containing the same foods and beverages consumed during the 3-day period of recording their habitual diet in amounts that provided DEI of 60 and 30 kcal·kg$^{-1}$LBM·d$^{-1}$ and maintained the dietary composition of each participant's habitual diet Three menus were designed according to the 3 records of habitual DEI and administered in a 3-day cyclic order with menu A on D 4 and D 7, menu B on D 5 and D 8 and menu C on D 6. Menus included five meals in both CON and RES trials to limit the effects of food partition on bone metabolism [28]. Participants were asked to consume these meals at standardised times each day; breakfast (08:30 h), mid-morning snack (10:00 h), lunch (12:00 h), afternoon snack depending on the time of exercise (15:00 or 17:00 h) and dinner (20:00 h). Participants provided their own food, but were instructed on the exact amounts, preparation and timing of the meals specified on their diet plans. Participants used their weighing scales during food preparation and consumption. Compliance with the experimental diets was confirmed verbally and/or by using a self-recorded checklist. Participants were allowed to change the order of the menus to enable them to adhere with the preparation of the diets. Some appropriate swaps were allowed after consultation with the dietitian of the study (e.g., banana instead of apple in amounts contributing to the same amount of calories). Participants were provided with one multivitamin, multi-mineral supplement (A - Z Tablets, Boots, Nottingham, UK. www.boots.com/en/boots-a-z-90-tablets_1289974/) during RES to maintain micronutrient intakes and isolate the effects of energy/macronutrient restriction. The multivitamin, multi-mineral supplement provided 5 µg Vitamin D (100% Nutrient Reference Value) and 200 mg Ca (25% Nutrient Reference Value). Supplementation was initiated on D 4 (main experimental protocol).

#### 2.3.2.4. EEE

During D 4 to 8, daily exercise was undertaken to expend 15 kcal·kg$^{-1}$LBM·d$^{-1}$. Participants ran at 70% of $VO_2$ peak in 15 minute sessions, with 5 minute rest periods between sessions. Expired gases were continuously collected and analysed using a breath-by-breath analyser (ZAN 600, nSpire Health, Germany). The required duration of exercise was determined using the oxygen uptake values and respiratory exchange ratio from D 4. Unpublished data from our laboratory have shown that EEE during exercise of the same duration and intensity is similar when repeated under the same conditions for the same individual. As such, following an initial measurement of EEE, exercise sessions were completed under under identical conditions to the first exercise day (D 4) in a supervised laboratory environment without the facemask and the

use of the breath-by-breath analysis system. Outside the prescribed exercise, participants were instructed to refrain from systematic exercise and perform only light activities, such as reading or working on a computer.

### 2.4. Blood sampling

Blood was obtained from a vein in the forearm by a trained phlebotomist using standardised procedures at the same time of day for each participant (08:15-09:00 h ± 15 min for the same participant) after an overnight fast (from 20:00 h the previous evening) on D 1, 3, 5, 7 and 9. Mean values of D 1 and 3 were used as the baseline (BASE) prior to each condition. Venous blood was collected into plasma tubes [ethylenediaminetetraacetic acid (EDTA) tubes, SARSTED, Nümbrecht, Germany], which were centrifuged immediately at 1509 x g at 4 $^{o}$C for 10 minutes, and serum tubes (Becton Dickinson Vacutainer System), which were allowed to clot at room temperature for 30 minutes before centrifuging at the same conditions. Plasma and serum samples were aliquoted into Eppendorf tubes and stored at -80$^{o}$C until analysis. β-carboxyl-terminal cross-linked telopeptide of type I collagen (β-CTX), amino-terminal propeptide of type 1 procollagen (PINP), parathyroid hormone (PTH) and IGF-1 were analysed in EDTA plasma and leptin, insulin, $T_3$, glucagon-like peptide-2 (GLP-2), 1*7β*-oestradiol, albumin, calcium (Ca), magnesium (Mg) and phosphate ($PO_4$) were analysed in serum.

### 2.5. Biochemical analysis

β-CTX, P1NP, PTH, $T_3$ and 1*7β*-oestradiol were measured using electro-chemiluminescence immunoassay (ECLIA) (Roche Diagnostics, Burgess Hill, UK) on a on a Cobas e601 . Inter-assay coefficient of variation (CV) for β-CTX was <3% between 0.2 and 1.5 μg·L$^{-1}$, with sensitivity of 0.01 μg·L$^{-1}$. P1NP inter-assay CV was <3% between 20-600 μg·L$^{-1}$ and sensitivity of 8 μg·L$^{-1}$. PTH inter-assay CV of <4% between 1-30 pmol·L$^{-1}$ and sensitivity of 0.8 pmol·L$^{-1}$. Sclerostin was measured using an enzyme-linked immunosorbent assay (ELISA) supplied by Biomedica GmbH (Vienna Austria) with a sensitivity of 2.6 pmol·L$^{-1}$ established from precision profiles (22% CV of duplicates) and a CV of <15% across the range 25-95.0 pmol·L$^{-1}$. $T_3$ inter-essay CV of <1% between 2.0-3.1 nmol·L$^{-1}$ and detection limit of 0.3 nmol·L$^{-1}$. The inter-assay CV for 1*7β*-oestradiol was <3% between 214.3-2156.7 pmol·L$^{-1}$ and detection limit of 18.4 pmol·L$^{-1}$. Leptin was measured using ELISA (Biovendor, Czech Republic) and had an inter-assay CV of <7% across the range 1-50 μg·L$^{-1}$ and sensitivity 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 sensitivity of 4.4 ng·mL$^{-1}$. GLP-2 was measured using ELISA

(Yanaihara Institute Inc, Japan), with an inter-assay CV of 1.1-11.1% across the range 3.1-33.4 ng·mL$^{-1}$ and a detection limit of 0.5 ng·mL$^{-1}$. Insulin was measured using ECLIA (Roche Diagnostics, Burgess Hill, UK), inter-assay CV is <6.1% across the range 44-505 pmol·L$^{-1}$ and sensitivity is 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 levels to change independently of the ionized 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 results reported by Zanker and Swaine (2000), the study was powered to detect a significant change in P1NP (pre: 76.1±5.8; post: 64.7±6.0 mg·L$^{-1}$, P<0.05) due to low EA [17]. An *a priori* power calculation determined that 9 women and 9 men were required to achieve 95% power at P<0.05 for each sex. Data were checked for normality using a Shapiro-Wilk test. Baseline characteristics for women and men were compared with paired t-test or Wilcoxon signed-rank test. Participant characteristics between women and men were compared using unpaired t-test or the Wilcoxon-rank sum test. The ratio between P1NP and β-CTX was calculated to provide a numerical quantification of bone turnover (BT ratio) as used previously [29]. An increase in the ratio would indicate a state of bone turnover favouring bone formation. Area under the curve (AUC) with respect to baseline (BASE) was calculated for all biochemical markers [30]. Single sex and between sex responses to EA were analysed. A paired t-test or Wilcoxon signed-rank sum test was used to detect differences between CON and RES for AUC in men and women. Data containing two factors (condition, sex) were analysed using a two-way repeated measures analyses of variance (ANOVA), with post hoc analyses performed using Tukey’s tests. Data are presented as mean ± 1 standard deviation (SD) and statistical significance was set at $P \leq 0.05$. Data were analysed using Statistica 13.0 (Statsoft, USA) and SPSS 22.0 (Armonk, USA). In addition to summary statistics, we also examined the individual responses of the BTM to RES. In order to be considered a responder, β-CTX concentrations at D 9 in RES were > BASE (100%), > β-CTX concentrations at D 9 in CON together with a difference

>3% to account for CV of β-CTX assay. For P1NP, responders were identified if P1NP concentrations at D 9 in RES were <BASE (100%), <P1NP levels at D 9 in CON together with a difference >3% to account for CV for P1NP assay.

## 3. Results

### 3.1. Baseline characteristics

There were no significant differences in habitual DEI, lifestyle EE, EA or body mass (D 4) between CON and RES for either women or men (data not shown). In women, baseline β-CTX in RES was significantly lower than baseline levels in CON ($P=0.01$), with no significant differences in any other biochemical marker (data not shown). In men, no significant differences were detected for any biochemical marker between baseline values before CON and RES, apart from leptin; with higher levels shown prior to RES compared to CON ($P<0.05$). Mean baseline concentrations were within, but at the higher end of the reference range (Women – β-CTX: 0.15-0.80 $\mu g \cdot L^{-1}$; P1NP: 25-90 $\mu g \cdot L^{-1}$; Men – β-CTX: 0.17-0.60 $\mu g \cdot L^{-1}$; P1NP: 15-80 $\mu g \cdot L^{-1}$) [31] for both β-CTX (Women: 0.49±0.14 $\mu g \cdot L^{-1}$; Men: 0.48±0.16 $\mu g \cdot L^{-1}$) and P1NP (Women: 71.1±15.0 $\mu g \cdot L^{-1}$; Men: 73.6±31.4 $\mu g \cdot L^{-1}$).

**Table 1.** Descriptive characteristics of physically active women (n=11) and men (n=11). (Size: 1 column)

| | **Women (n=11)** | **Men (n=11)** |
|---|---|---|
| **Demographics** | | |
| **Age (y)** | 26±5 | 26±5 |
| **Height (m)** | 1.66±0.05 | 1.78±0.07** |
| **Body mass (kg)** | 59.7±6.7 | 73.1±8.0** |
| **BMI (kg ·m$^2$)** | 21.5±1.5 | 23.0±1.6* |
| **Body composition** | | |
| **Body fat (%)** | 27.0±6.2 | 18.3±3.4** |
| **Lean body mass (kg)** | 41.50±4.91 | 57.23±7.32** |
| **Fat free mass (kg)** | 43.95±5.14 | 60.33±7.58** |
| **BMD (g·cm$^{-2}$)** | 1.14±0.11 | 1.25±0.08* |
| **Training characteristics** | | |
| **$VO_2$ peak (ml·kg$^{-1}$·min$^{-1}$)** | 47.9±5.5 | 54.2±5.3* |
| **$VO_2$ peak (ml·kg LBM$^{-1}$·min$^{-1}$)** | 68.7±4.0 | 69.3±5.9 |
| **Physical activity (MET-min·wk$^{-1}$)** | 3927±1651 | 3443±1006 |
| **Dietary and energy expenditure characteristics** | | |
| **Habitual DEI (kcal·d$^{-1}$) [a]** | 2143± 361 | 2682± 265** |
| **Habitual DEI (kcal·kgLBM·d$^{-1}$) [a]** | 51.9 (9.2) | 47.2 (4.7) |
| **Lifestyle Energy Expenditure (kcal·d$^{-1}$) [a]** | 402± 227 | 455±136 |
| **Lifestyle Energy Expenditure (kcal·kgLBM$^{-1}$·d$^{-1}$) [a]** | 9.5 (5.1) | 8.0 (2.6) |
| **Habitual EA (kcal·kgLBM$^{-1}$·d$^{-1}$) [a]** | 51.9 (9.2) | 47.2 (4.7) |

[a] Mean values of D 1 to D 3 prior to both experimental conditions.Values are presented as means±1SD. * denotes a significant difference from women ($P<0.05$), ** denotes a significant difference from women ($P\leq0.001$).

BMI, Body mass index; BMD, Bone mineral density; LBM, Lean Body Mass, $VO_2$peak, Peak aerobic capacity; MET, Metabolic equivalent; DEI, Dietary Energy Intake; EA, Energy availability.

### 3.2. Actual experimental diets and exercise

The actual characteristics of experimental diets and exercise session are described in Table 2. All participants completed the running sessions and expended the prescribed EEE as measured on D 4.

**Table 2.** Actual experimental dietary and exercise characteristics for women and men in CON and RES

| | **Women** | | **Men** | |
|---|---|---|---|---|
| | CON | **RES** | **CON** | **RES** |
| | **Actual Experimental Dietary characteristics** | | | |
| **DEI (kcal· $d^{-1}$)** | 2465±299 | 1261±125 | 3383±393 | 1720±235 |
| **DEI (kcal·$kg^{-1}$ LBM·$d^{-1}$)** | 59.4±1.4 | 30.5±0.8 | 59.2±1.1 | 30±0.2 |
| **Carbohydrate (%)** | 49±8 | 49±8 | 47±8 | 48±9 |
| **Protein (%)** | 17±5 | 18±5 | 18± 3 | 19±4 |
| **Fat (%)** | 34±7 | 33±6 | 35±7 | 33±7 |
| | **Actual Experimental Exercise** | | | |
| **EEE (kcal· $d^{-1}$)** | 616±74 | 616±74 | 856±110 | 856±110 |
| **EEE (kcal·$kg^{-1}$ LBM·$d^{-1}$)** | 14.8±0.2 | 14.8±0.2 | 15.0±0.1 | 15.0± 0.1 |
| **Running speed (km·$h^{-1}$)** | 9.0± 1.6 | 9.0±1.6 | 10.5± 1.7 | 10.5±1.7 |
| **Duration (min)** | 66±4 | 66±4 | 65± 7 | 65±7 |

Values are presented as means±1SD.

DEI: Dietary Energy Intake; EEE: exercise energy expenditure; LBM: Lean body mass; CON: Controlled; RES: Restricted.

### 3.3. Body mass

Body mass was maintained in CON (between D 4 and D 9) (Women: 59.9±6.5- 59.7± 6.5 kg, P=0.15 or -0.2±0.4% at D 9 from D 4; Men: 72.7±8.1- 72.9±8.1 kg, P=0.31; 0.2±0.4% at D 9 from D 4), but was significantly decreased by -2.7%±0.6 and -2.5±0.8% at D 9 from D 4 in women and men in RES (Women: 60.2±6.2- 58.6±5.9 kg, P<0.001; Men: 73.0 ±8.1- 71.2 ±7.9 kg; P<0.001) in women and men.

### 3.4. BTMs

**β-CTX:** In women, there was an increase in β-CTX in RES when compared to CON, as demonstrated by a greater AUC (P=0.03). In men, the difference in β-CTX AUCs between RES and CON was not significant (P=0.46). Changes in β-CTX appeared to differ between men and women, as indicated by a significant sex x condition interaction (P=0.03), although post hoc comparisons did not confirm significant differences among men and women during RES (P=0.59) or CON (P=0.64) (Figure 2).

**P1NP:** In women, a lower P1NP AUC (P=0.01) in RES compared to CON suggests a decrease in P1NP with low EA. In men, the difference in P1NP between RES and CON was not significant (P=0.12). For pooled men and women data, PINP responses were lower in RES than in CON (condition main effect, P=0.008). P1NP responses did not differ between men and women, as indicated by a non-significant main effect of sex (P=0.27) or a sex x condition interaction effect (P=0.90) for P1NP AUC (Figure 2).

**BT ratio:** In women, a lower BT ratio AUC (P=0.01) was noted in RES compared to CON, suggesting a reduction in bone metabolism with low EA In men, the difference in P1NP between RES and CON was not significant (P=0.33). For pooled men and women data, BT ratio responses were lower in RES than in CON (condition main effect, P=0.008). BT ratio responses were not differerent between sexes, as indicated by a non-significant main effect of sex (P=0.48) or a sex x condition interaction (P=0.57) for BT ratio AUC (Figure 2).

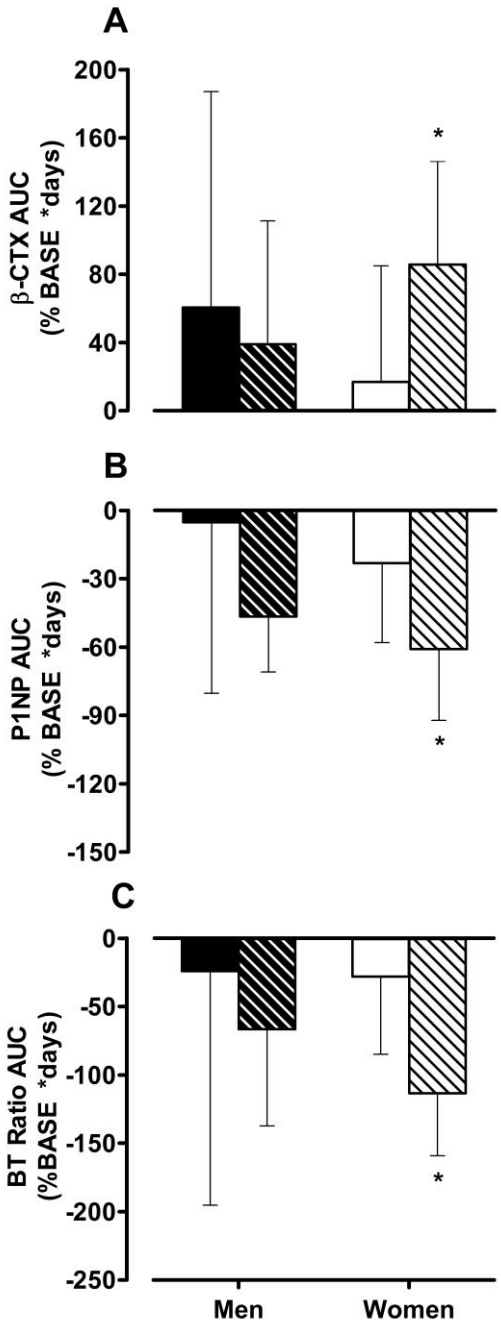


**Figure 2.** AUC analysis of β-CTX (A), P1NP (B) and BT ratio (C) in men (black bars) and women (white bars) in CON (solid colour) and RES (stripe pattern). Values are presented as means±1SD. * denotes a significant difference from CON trial ($P<0.05$). (Size: 1 column)

β-CTX, β-carboxyl-terminal cross-linked telopeptide of type I collagen; P1NP, Amino-terminal pro-peptide of type 1 procollagen; BT Ratio, Bone turnover ratio; D, Day; BASE, Baseline; AUC, Area under the curve; CON, Controlled trial; RES, Restricted trial.

### 3.5.. Markers of calcium metabolism

PTH, ACa, Mg and $PO_4$ AUCs in RES were not significantly different from AUC in CON in women or men (P values >0.05) and no significant sex differences in response to RES were shown (P values >0.05).

### 3.6. Regulatory and Reproductive Hormones

**Sclerostin:** Sclerostin AUC in RES was not significantly different from AUC in CON in women (P=0.48) or men (P=0.31). Overall, responses in sclerostin appeared to be higher in women compared to those in men, as suggested by a significant main effect of sex (P=0.01) (Table 2).

**IGF-1:** IGF-1 AUC in RES was not significantly different from AUC in CON in women (P=0.09) or men (P=0.18). For pooled men and women data, IGF-1 responses were lower in RES than in CON (condition main effect, P=0.02). Overall, IGF-1 responses appeared to be lower in women than in men, as suggested by a significant main effect of sex (P=0.04) (Table 2).

**$T_3$**: In either women or men, $T_3$ AUCs between RES and CON were not significantly different (P=0.054 and P=0.09). For pooled men and women data, $T_3$ responses were lower in RES than in CON (condition main effect, P=0.008). Overall, $T_3$ responses did not appear to differ between men and women, as suggested by a non-significant main effect of sex (P=0.18) or sex x condition interaction fot $T_3$ AUC (P=0.054) (Table 2).

**Leptin:** In women, a decrease in leptin was shown with low EA, as demonstrated by a lower leptin AUC (P=0.01) in RES compared to CON. In men, leptin AUCs between RES and CON were not significantly different (P=0.07). For pooled men and women data, leptin responses were lower in RES than in CON (condition main effect, P=0.008). Overall, leptin responses appeared to differ between men and women, as indicated by a significant main effect of sex for leptin AUC (Women<Men) (P=0.02).

**Insulin:** In women, a lower insulin AUC (P=0.01) in RES compared to CON suggests a reduction in insulin with low EA In men, the difference in insulin AUCs between RES and CON was not significant (P=0.40). For pooled men and women data, insulin responses were lower in RES than in CON (condition main effect, P=0.02). Insulin responses did not differ between men and women, as indicated by a non-significant main effect of sex (P=0.25) or a sex x condition interaction for insulin AUC (P=0.28) (Table 2).

**GLP-2:** GLP-2 AUC in RES was not significantly different from AUC in CON in women (P=0.14) or men (P=0.51). GLP-2 responses did not differ between sexes, as indicated by a non-significant main effect of sex (P=0.29) or a sex x condition interaction for insulin AUC (P=0.80) (Table 2).

**Reproductive hormones:** In women, the difference in 17β-oestradiol AUC between RES and CON was not significant (P=0.39) (Table 2).

**Table 2.** AUCs (%BASE x days) for BTMs, markers of calcium metabolism, regulatory and reproductive hormone in CON and RES trials. (Size: 2 columns)

| | **Women (n=11)** | | **Men (n=11)** | |
|---|---|---|---|---|
| | **CON** | **RES** | **CON** | **RES** |
| **Markers of Calcium Metabolism** | | | | |
| **PTH** | 49.9±59.4 | 76.1±82.6 | 65.9± 121.9 | -3.2± 97.4 |
| **ACa** | -0.9±5.9 | 1.0±6.8 | -7.9±8.3 | -1.0±8.1 |
| **Mg** | 2.5±17.6 | 10.1±21.3 | 6.0±19.7 | -8.2±63.8 |
| **$PO_4$** | 17.0±36.9 | 18.9±36.1 | 14.5±73.8 | -23.1±33.1 |
| **Regulatory and Reproductive Hormones** | | | | |
| **Sclerostin** | 28.8±81.6 | 3.1±27.8 | 18.3±55.6 | 37.4±52.6 |
| **IGF-1** | -48.2±82.6 | -90.9±47.2 | 9.4±96.9 | -54.0± 48.1 |
| **$T_3$** | -1.8±61.3 | -31.5±40.5 | -6.4±41.0 | -41.4± 47.6 |
| **Leptin**[a] | -118.3±119.0 | -217.1±113.0[*] | 158.9±219.7 | -26.7±331.1 |
| **Insulin** | -16.5±194.2 | -180.5±126.6[*] | 23.0± 241.3 | -40.5±239.5 |
| **GLP-2** | -20.2±70.5 | 9.6±50.8 | -27.0±60.2 | -6.3±50.3 |
| **17β-oestradiol** [b,c] | 119.0±108.7 | 78.5±63.0 | - | - |

AUC was calculated for each experimental condition from BASE to D 9.Mean values of D 1 and 3 were used as BASE prior to each experimental condition. Values are presented as means±1SD. [*]denotes a significant difference from CON trial ($P<0.05$). [a]Leptin was undetectable in 6 male participants; therefore, the analysis was performed on the remaining 5 men. [b]17β-oestradiol levels were determined at D 5 and D 9 in women only. [c]Analysis performed in 9 participants with available data in both trials.

PTH, Parathyroid hormone; Mg, Magnesium; ACa, Albumin adjusted Calcium; $PO_4$, Phosphate; IGF-1, Insulin-like growth factor 1; $T_3$, Triiodothyronine; GLP-2, Glucagon-like peptide 2; AUC, Area under the curve; D, Day; BASE, Baseline; CON, Controlled trial; RES, Restricted trial.

### 3.7. Individual analysis

Individual responses were considered in line with the criteria outlined in the statistical methods. In women, seven out of eleven participants responded to RES with an increase in β-CTX concentrations and six out of eleven participants responded to RES with a decrease in P1NP concentrations. Three out of eleven female participants were responders to RES for both β-CTX (increase) and P1NP (decrease). Three out of eleven men responded to RES with an increase in β-CTX concentrations and six out of eleven men responded to RES with a decrease in P1NP concentrations. Three out of eleven male participants were responders to RES for both β-CTX (increase) and P1NP (decrease). In total, six out of eleven men and ten out eleven women had altered bone turnover resulting from changes in bone resorption (increase), bone formation (decrease) or both (Table 3).

**Table 3.** Number of responders (out of total number of participants) for β-CTX, P1NP and bone turnover. This analysis was based on data expressed as %BASE for each participant in RES trial. (Size: 2 columns)

| | ***β*-CTX** | **P1NP** | **Bone turnover[a]** | **Bone turnover[b]** |
|---|---|---|---|---|
| **Women (n=11)** | 7/11 | 6/11 | 3/11 | 10/11 |
| **Men (n=11)** | 3/11 | 6/11 | 3/11 | 6/11 |
| **Total (n=22)** | 10/22 | 12/22 | 6/22 | 16/22 |

[a]altered bone turnover due to a simultaneous increase in *β*-CTX and decrease in P1NP.

[b]altered bone turnover due to an increase *β*-CTX (only), decreased P1NP (only) or both.

β-CTX, β-carboxyl-terminal cross-linked telopeptide of type I collagen; P1NP, Amino-terminal pro-peptide of type 1 procollagen; BT ratio, Bone turnover ratio; CON, Controlled trial; RES, Restricted trial.

## 4. Discussion

We investigated the impact of short-term, low EA at 15 kcal·kgLBM$^{-1}$·d$^{-1}$ on bone metabolism in women and men, and then compared responses between sexes. Our main findings were 1) a significant increase in bone resorption and a decrease in bone formation in response to low EA in women, 2) no significant effect of low EA on either bone formation or resorption in men and 3) no significant differences in bone metabolic responses between sexes, despite altered bone metabolism in women with low EA.

Our findings in women extend those of previous studies documenting decreased bone formation with or without an increase in bone resorption with low EA [15] and acute fasting [32]. The increases in bone resorption (β-CTX: +19%) shown at 15 kcal·kgLBM$^{-1}$·d$^{-1}$ occured in the same direction as the changes documented at 10 kcal·kgLBM$^{-1}$·d$^{-1}$ [urinary amino-terminal cross-linked telopeptide of type I collagen (NTX): +34%] by Ihle & Loucks (2004) [15]. The reduction in bone formation (P1NP: -13%) was also similar to that induced by an EA of 30 kcal·kgLBM$^{-1}$·d$^{-1}$, previously shown with exercise and dietary manipulations [15]. We cannot, however, provide a more direct comparison of the magnitude of these effects between the two studies since different BTMs were used [*i.e.*, β-CTX *vs.* NTX and P1NP vs carboxyl-terminal propeptide of type 1 procollagen (P1CP)] and in different samples (*i.e.*, plasma and urine). β-CTX and P1NP analysed in blood samples have been chosen as the reference markers for bone resorption and formation [33]. Conversely, urinary NTX (bone resorption) and P1CP (bone formation) are limited by analytical variability and thus, are not included in recent recommendations about reference BTMs, published by expert scientific bodies in the area of bone health and disease [33]. Evidence underpinning the recommendations on the preventation and treatment of the Female Athlete Triad are based largely on the bone metabolic responses of sedentary women to EA [15]. We cannot assume that what we know for sedentary individuals holds true for physically active individuals. Sedentary and trained participants may differ in body composition, habitual physical activity, resting BTM levels, and bone strength from athletic individuals [34-36] and bone is accustomed to exercise-induced loading in physically active individuals. For these reasons, the bone metabolic response to low EA may also differ to physically active populations. The level of low EA chosen to represent the EA of some amenorrheic athletes, who are at high risk for bone injuries [2, 16], making the current results relevant to regular exercisers.The baseline responses in our physically active population were towards the higher end of the reference range (in line with other data from our laboratory on similar populations) suggesting that the physically active women in our

study had higher bone turnover than non-active populations, with bone resorption increasing and bone formation decreasing in response to RES.

A 24% reduction in BT ratio in response to RES, but not CON indicates a change in bone turnover in favour of bone resorption. These responses could, if continued over time (*e.g.*, in individuals with anorexia nervosa), result in a net bone loss, which could adversely affect bone health and increase the risk of bone injury [37] by inducing changes in bone volume, mineralisation of the bone matrix, collagen cross-linking and the appearance of remodelling cavities resulting in poorer bone quality [38]. The time course of such changes warrant further investigation.

Sclerostin is secreted by osteocytes and acts as a Wnt antagonist that, through the Wnt/β-catenin pathway (signalling pathway involved in bone cell activation), regulates osteoblast activity [39] and therefore, bone formation. The absence of changes in sclerostin following RES within women may suggest that the observed reduction in P1NP is not mediated by sclerostin. Sclerostin is highly responsive to changes in mechanical loading [40] and exercise during weight loss prevents the increase in sclerostin [41], which might explain the lack of any effect on sclerostin in the current study given that participants completed identical running protocols (≥1hour) and were exposed to the same amount of mechanical loading in both CON and RES.

Lower leptin and insulin responses in women in RES compared to CON, are consistent with previous studies investigating energy deprived conditions [15, 18] and indicate adaptations for sparing energy for vital functions [1]. Alterations in these hormones may have mediated the changes in BTM. Leptin exerts direct and indirect actions on bone metabolism that are generally protective of BMD-for a review see [42]. Insulin is a potential determinant of BMD [43] and insulin deficiency is often accompanied by reduced BMD and increased fragility risk [44, 45], although this seems to have an effect on specific skeletal sites rather than promoting general bone loss. $T_3$ effects on bone may be exerted either directly via thyroid hormone receptors in bone, or through indirect regulation of the GH/ IGF-I axis [46], although these effects seem unlikely in the current study given that $T_3$ or IGF-1 AUCs were not altered in response to reduced EA in women. We did not show any changes in 17β-oestradiol concentrations, possibly due to our short-term intervention and single measurement of 17β-oestradiol levels. Loucks and colleagues reported a 15% reduction in pooled 24-h mean

oestrogen concentrations, which occurred in parallel with an increase in bone resorption (urinary NTX) following 5 days of low EA attained through diet and exercise at 10 kcal·kgLBM$^{-1}$·d$^{-1}$, but not 20 kcal·kgLBM$^{-1}$·d$^{-1}$ [15]. The discrepancies between the studies may be in part due to our less severely reduced EA (15 vs. 10 kcal·kgLBM$^{-1}$·d$^{-1}$) or blood sampling schedule (single sample vs. 24-h frequent blood collection) [15].

BTMs in men were not significantly affected by reduced EA. P1NP response in RES (-14% from BASE), although did not reach statistical significance, is similar to the significant 15% reduction in P1NP shown in eight male runners under conditions of energy deprivation by Zanker & Swaine (2000) [17]. Possible reasons why we did not show the same BTM responses in men as in women are that a more severe level of EA or a more prolonged exposure to low EA is needed to elicit an EA-related change in BTM in men. Available cross-sectional studies in male athletes (reviewed in [4]) suggest that low BMD is mainly confined to those partaking in weight sensitive sports and experiencing multiple low EA-related risk factors including low BMI, repeated bouts of rapid weight loss or disordered eating. Although the prevalence rates of low BMD and low EA in men and how these compare to the rates of these conditions in women remain unkwnon, the prevalence rates of stress fracture injury have been reported to be lower in males than their female counterparts [47].

Bone formation and/or bone resorption were altered due to low EA in some men, in a direction favouring bone loss, while others remained unaffected, indicating that it might be premature to suggest that low EA does not impact bone turnover in all men. Six out of eleven men had altered bone turnover resulting from changes in either bone formation, bone resorption or both. Our study population was representative of physically active individuals and strict inclusion and exclusion criteria were applied to eliminate confounding factors including age and training status [48]. It is unlikely that the variability in men is attributable to these factors. Some of the observed inter-individual variability might be accounted for by genetic differences, which have previously been associated with bone health [49] and stress fracture injury [50]. More consistent BTM responses were reported in women with ten out eleven women having altered bone turnover resulting from changes in either bone formation, bone resorption or both. These results are suggestive of inter-individual variability in susceptibility/sensitivity to low EA, which may be sex-specific given the more consistent responses among our female participants. Inter-individual variability to energy deficiency has been reported in body composition changes [51] and female reproductive function [52]; which may be relevant to sex-specific bone turnover responses.

No differences in regulatory hormones responses (sclerostin, IGF-1, leptin, $T_3$, insulin or GLP-2) were shown between conditions in men perhaps explaining why there was no significant effect of low EA on BTM responses. Previous studies have shown significantly decreased leptin and insulin using the same level of low EA [53], although the magnitude of the responses to low EA was similar to that shown in the current study. In agreement with our findings, other short-term energy deprivation studies have not shown changes in leptin [20] or $T_3$ concentrations [20, 53]. IGF-1 did not change over time in response to low EA within men in the current study, constrasting the findings reported previously [17, 23]. The anabolic effects of IGF-1 on bone involve the stimulation of osteoblast differentiation, expression of type I collagen [54] and suppression of transcriptional factors that contribute to collagen breakdown [55]. The absence of changes in regulatory hormones support the BTM responses within the 5-day timeframe of the present study.

The direct between sexes comparisons showed no significant differences in BTM AUCs following low EA. The magnitudes of the BTM responses were similar between men and women (Men - β-CTX: +12%, P1NP: -14%; BT ratio:-21%; Women - β-CTX: +19%, P1NP: -13%; BT ratio:-24%). This suggests the greater inter-individual variability in BTM responses within men may have masked any statistical significance. When we directly compared the responses of regulatory hormones to low EA, there were no significant differences between women and men for any of the hormones measured. The evidence from human research is contradictory, with some studies opposing [19, 20], and others supporting [56] our findings.

In the current investigation, EA was manipulated by altering habitual diet in order to limit possible initial adaptations to macronutrient intake and mimic their daily lives. Ihle and Loucks (2004) utilised clinical dietary products in liquid form, which provide the advantage of uniform dietary prescription, but are likely to alter regulatory hormones (compared to solid habitual food) [57] that may, in turn, affect bone metabolism. Small deviations were reported in dietary energy intake in both the controlled (target; 60 kcal· $kgLBM^{-1}\cdot d^{-1}$, range 57.7-61.0 kcal· $kgLBM^{-1}\cdot d^{-1}$) and restricted EAs (target: 30 kcal· $kgLBM^{-1}\cdot d^{-1}$, actual range 28.5-31.3 kcal· $kgLBM^{-1}\cdot d^{-1}$). This study was designed to assess the effects of low EA achieved by dietary energy restriction and exercise and not specific macronutrient restriction. Although the absolute macronutrient composition was similar within participants in both experimental conditions, it is possible that differences

in relative dietary intake of macronutrients, such as protein, may have influenced the observed results. Restriction of bone-related micronutrients, including calcium and Vitamin D, may also alter bone metabolic responses. To minimise this influence, our participants were provided with a multivitamin, multi-mineral supplement in the restricted condition. Indeed, we did not show any effects of low EA on PTH, ACa, Mg or $PO_4$ levels in response to RES, making it unlikely that the alterations on bone turnover in women were mediated by changes in these factors. Some previous experiments in the area of energy restriction and bone health have failed to consider micronutrient restriction accompanying energy restrictions [17], while some others provided a multivitamin, multimineral supplement [15] similar to the current investigation.

## 5. Conclusions

Reduced EA at 15 kcal·kgLBM$^{-1}$·d$^{-1}$ decreased bone formation and increased bone resorption in women, which was accompanied by decreased insulin and leptin responses compared to controlled EA. No effects of low EA were shown in men, but when considering individual responses, some individuals adversely responded to low EA. Despite the fact that the sample size used was based upon preliminary data, some responses did not reach statistical significance in our male cohort at least within the 5-day timeframe of the current investigation.. When comparing directly, however, no significant between sexes differences were shown, with the magnitude of the changes in BTMs being similar in men and women. As such, our findings in women support previous studies showing that low EA is an important mediator of bone health in women [2, 3, 7]. Our data in men may suggest an analogous relationship with women when considering the similar BTM responses shown in this study between men and women to low EA. Larger, longer-term studies should confirm these findings in future investigations.



**Confict of interest**

None

**Acknowledgements**

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

**Funding sources**

The study formed part of a programme of work for M.P., which was supported by a Vice Chancellors Research Student Bursary from Nottingham Trent University.

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**Highlights**

- The effects of low energy availability (EA) on bone turnover markers (BTMs) in physically active women and men were explored.
- In women, reduced EA at 15 kcal·kgLBM$^{-1}$·d$^{-1}$ resulted in decreased bone formation and increased bone resorption.
- Overall, reduced EA at 15 kcal·kgLBM$^{-1}$·d$^{-1}$ did not impact any BTM in men.
- The high inter-individual variability in men suggests that BTMs changed following reduced EA in some men, but not in others.
- There were no sex differences in BTM responses to reduced EA, with their magnitude being similar in men and women.