NOTTINGHAM TRENT UNIVERSITY

The Effects of Energy Availability on Bone Metabolism and Health

Maria Papageorgiou

Thesis submitted in partial fulfilment of the requirements of Nottingham Trent University for the degree of Doctor of Philosophy

September 2016

Abstract

No experimental studies have been conducted on the impact of low EA on bone metabolism in relevant physically active populations. We evaluated the effects of low EA on bone turnover markers (BTMs) in a cohort of Caucasian, physically active eumenorrheic women **(Study 1)** and men **(Study 2)**, and compared effects between sexes (sex comparison). These two studies were performed using a randomised, counterbalanced, crossover design. Eleven eumenorrheic women [peak aerobic capacity (VO_2 peak): 47.9±5.5 ml·kg^{-1}·min^{-1}] and eleven men (VO_2 peak: 54.2±5.3 ml·kg^{-1}·min^{-1}) completed two 5-day protocols; controlled (CON; 45 kcal·kgLBM^{-1}·d^{-1}) and restricted (RES; 15 kcal·kgLBM^{-1}·d^{-1}) EAs. Participants ran daily under supervision on a treadmill at 70% of their VO_2 peak resulting in an exercise energy expenditure (EEE) of 15 kcal·kgLBM^{-1}·d^{-1} and consumed diets providing 60 and 30 kcal·kgLBM^{-1}·d^{-1} (based on participants' habitual dietary composition). The effects of short-term (3 days) low EA induced by diet or exercise alone on BTMs in Caucasian, physically active eumenorrheic women **(Study 3)** and oral contraceptive (OCP) users were explored **(Study 4)**, and compared effects between groups **(comparison between eumenorrheic women and combined OCP users)**. Studies 3 and 4 were performed using a randomised, counterbalanced, crossover design. Ten eumenorrheic women (VO2 peak: 48.1 ±3.3 ml·kg^{-1}·min^{-}1) and 10 combined OCP users (VO2 peak: 47.9±5.5 ml·kg^{-1}·min^{-1}) completed three 3-day protocols; controlled (CON; 45 kcal·kgLBM^{-1}·d^{-1}) and restricted through diet (D-RES; 15 kcal·kgLBM^{-1}·d^{-1}) and restricted through exercise (E-RES; 15 kcal·kgLBM^{-1}·d^{-1}) EAs. In CON, D-RES and E-RES participants consumed diets providing 45, 15 and 45 kcal·kgLBM^{-1}·d^{-1} with standardised composition (50% carbohydrates, 20% protein and 30% fat) in all experimental conditions. In E-RES only, participants completed supervised laboratory exercise sessions at an exercise intensity of 70% of their VO_2 peak that resulted in an EEE of 30 kcal·kg LBM^{-1}·d^{-1}.

Study 1: Short-term low EA at 15 kcal·kgLBM^{-1}·d^{-1} decreased bone formation [Amino-terminal propeptide of type 1 procollagen (P1NP) AUC; $P<0.05$] and increased bone resorption [Carboxyl-terminal cross-linked telopeptide of type I collagen (β-CTX) AUC; $P<0.05$] in physically active, eumenorrheic women. Significant reductions in energy regulatory hormones; leptin, insulin and triiodothyronine (T_3), but no changes in reproductive hormones or markers of calcium metabolism were shown. **Study 2:** In men, low EA at 15 kcal·kgLBM^{-1}·d^{-1} had no significant effects on bone formation or bone resorption. Insulin growth factor 1 (IGF-1) was significantly reduced ($P<0.05$), whereas regulatory hormones or markers of calcium metabolism were not affected. **Sex comparison:** No significant differences were shown between sexes, with the magnitude of the responses

in all markers to low EA being similar in men and women. **Study 3:** Short-term diet-induced low EA (15 kcal·kgLBM^{-1}·d^{-1}) resulted in a reduction in bone turnover (BT) ratio ($P<0.05$), despite no alterations in P1NP and β-CTX in eumenorrheic women. Exercise-induced low EA did not impact BTM responses. Between diet- and exercise-induced low EAs there were no differences in BTM responses. Both low EA were accompanied by reductions in IGF-1 and leptin; T_3 was reduced following diet-induced low EA only and insulin decreased in exercise-induced low EA only. **Study 4:** Neither P1NP nor β-CTX were affected by low EA in combined OCP users. These effects were not different depending on whether low EA was diet- or exercise-induced. IGF-1 was reduced in the exercise-induced low EA only, T_3 decreased following diet-induced low EA only and leptin decreased in response to both low EAs. The **direct comparison between combined OCP users and eumeorrheic women** revealed no differences in BTM responses to either diet- or exercise-induced low EA.

These findings suggest that short-term low EA (achieved through a combination of dietary restriction and exercise) is critical for bone metabolism and health in physically active women, supporting the Female Athlete Triad and the RED-S models. Although our study in men does not support the RED-S models within the short timeframe of this study, our direct sex comparison suggests a similar relationship between low EA and bone metabolism in men EA relative to women. Low EA through diet or exercise does not affect bone metabolism, despite a reduction in regulatory hormones. Combined OCP use does not affect bone metabolism and health following short-term low EA. When compared to eumenorheic women, combined OCP users show similar BTM responses when exposed to the same dietary and exercise conditions.

List of Abbreviations

1,25-$(OH)_2$ D	1,25-dihydroxyvitamin D
25(OH)D	25-hydroxyvitamin D
ACa	Albumin-adjusted Calcium
ACSM	American College of Sport Medicine
ANOVA	Analysis of variance
AR	Androgen receptor
BALP	Bone specific alkaline phosphatase
BASE	Baseline
BMC	Bone mineral content
BMD	Body mineral density
BMI	Body mass index
BMP	Bone morphogenic proteins
BT ratio	Bone turnover ratio
BTM	Bone turnover markers
c-fms	Colony stimulating factor 1 receptor
CON	Controlled
CTX	Carboxyl-terminal cross-linked telopeptide of type I collagen
CV	Coefficient variation
DEI	Dietary energy intake
DKK-1	Dickkopf 1
DMPA	Depot medroxyprogesterone acetate
DMP-1	Dentin matrix acidic phosphoprotein 1
DPD	Deoxypyridinoline
D-RES	Diet-induced restricted
DXA	Dual energy x-ray absorptiometry
EA	Energy availability
ECLIA	Electro-chemiluminesence immunoassay
EDTA	Ethylenediaminetetraacetic acid
EE	Energy expenditure
EEE	Exercise energy expenditure
ELISA	Enzyme linked immunosorbent assay
ER	Estrogen receptor
E-RES	Exercise-induced restricted
FFM	Fat free mass
FHA	Functional hypothalamic amenorrhea

FSH	Follicle stimulating hormone
GH	Growth hormone
GLP-1 and 2	Glucagon-like- peptide-1 and -2
Glu	Glutamic
GnRH	Gonadotropin releasing hormone
GSK-3β	Glycogen synthase kinase 3
HR-MRI	High-resolution magnetic resonance imaging
HR-pQCT	High-resolution peripheral quantitative computed tomography
ICC	Interclass Correlation
IGF-1 and 2	Insulin-like growth factor 1 and 2
IL	Interleukin
LBM	Lean body mass
LH	Luteinizing hormone
LRP	Low-density lipoprotein receptor-related protein
M-CSF	Macrophage colony-stimulating factor
MET	Metabolic equivalents
MRI	Magnetic resonance imaging
NTU	Nottingham Trent University
NTX	Amino- terminal telopeptide of type I collagen
ob gene	Obese gene
OC	Osteocalcin
OCP	Oral contraceptive pill
OPG	Osteoprotegerin
Osx	Osterix
P1CP	Carboxyl-terminal propeptide of type 1 procollagen
P1NP	Amino-terminal propeptide of type 1 procollagen
PO_4	Phosphate
pQCT	Peripheral quantitative computed tomography
PTH	Parathyroid hormone
PYD	Pyridinoline
PYY	Peptide YY
QCT	Quantitative computed tomography
RED-S	Relative energy deficiency in sports
RLoA	Ratio of limits of agreement
RMR	Resting metabolic rate
RANK	Receptor activator of nuclear factor kappa B
RANKL	Receptor activator of nuclear factor kappa B ligand

RCT	Randomised controlled trial
RES	Restricted
RMR	Resting metabolic rate
Runx2	Runt-related transcription factor
SCOFF	Sick, Control, One stone, Fat, Food
SD	Standard Deviation
SHBG	Sex hormone binding globulins
SOST	Sclerostin encoding gene
SPSS	Statistics package for social science
T_3	Triiodothyronine
T_4	Thyroxine
TEE	Total energy expenditure
TGF-β	Transforming growth factor β
TNF-α	Tumour necrosis factor α
TRACP5b	Tartate-resistant acid phosphatase 5b
VO_{2peak}	Peak Oxygen Uptake
VLCD	Very low calorie diet
Wnt	Wingless
β-CTX	Carboxyl-terminal cross-linked telopeptide of type I collagen

Manuscripts

Papageorgiou, M., Elliott-Sale, K.J., Parsons, A., Tang, J.C.Y., Greeves, J.P., Fraser, W.D. and Sale C., 2016. Effects of reduced energy availability on bone metabolism in men and women. *Med Sci Sports Exerc* (under review).

Conference communications

Papageorgiou, M., Elliott-Sale, K.J., Greeves, J.P., Fraser, W.D. and Sale, C., 2016. Impaired bone turnover in women, but not in men, in response to low energy availability. American College of Sports Medicine, Boston.

Martin, D.E., **Papageorgiou, M.**, Colgan, H., Fraser, W.D., Greeves, J.P., Sale, C., Cooper, S.B. and Elliott-Sale, K.J., 2016. Effect of reduced energy availability by either diet or exercise on muscle force. American College of Sports Medicine, Boston.

Papageorgiou, M., Elliott-Sale, K.J., Greeves, J.P., Fraser, W.D. and Sale, C., 2015.The effects of short-term energy availability on bone turnover in women. European College of Sports Medicine, Malmo.

Papageorgiou, M., Elliott-Sale, K.J., Greeves, J.P., Fraser, W.D. and Sale, C., 2015. The effects of short-term energy availability on bone turnover in men. Highlighted Symposium-Nutrition for Bone Health in Athletes. American College of Sports Medicine, San Diego.

Acknowledgements

I would like to thank my director of studies, Professor Craig Sale, for providing me the opportunity to embark on this PhD programme, showing faith in my abilities as a research student and supporting me throughout the programme. My gratitude to Dr Elliott-Sale who has shared with me her passion for women's health research and provided her advice throughout this programme, but also to Dr Julie Greeves and Professor William Fraser who have assisted with study design and have contributed to this programme with invaluable suggestions.

I would like to thank Dr Jonathan Tang (University of East Anglia) for conducting the biochemical analysis for the studies of this programme, Mr Alan Parsons (University of Derby) for performing DXA scans (Study 1 and Study 2) and Mr Terrace Campion for his help with participants' transfer to University of Derby.

I would also like to thank past and current postgraduate students and staff in the Sports Department for their advice and enjoyable breaks throughout these years. I must thank Mr Daniel Martin for putting up with me during our collaborative studies, I am sure we will remember the early mornings and demanding weekends we devoted to these studies, but also the interesting people we collaborated with. Notably, in the collaborative studies with Mr Martin, in addition to bone outcomes presented in this thesis, we also conducted measurements of muscle and cognitive function. The results of these measurements will be presented in his PhD thesis.

I should also thank all the participants who "starved" for the studies in this programme. The different personalities made each session an adventure and some great friendships were developed.

I would like to thank Dr Tzortzis Nomikos (Harokopio University) who has contributed greatly to my passion for research as my mentor during my undergraduate project and has supported me since then. Many thanks to Dr Liz Simpson (University of Nottingham) who provided me the opportunity to be part of a unique team and get further experience in a great research programme.

Special thanks to my friends, Anna and Estefania, for always being there for me, for getting unique experiences in Nottingham and sharing life moments. Iro, Maria, Susan and Edwin, I am lucky to be part of this adorable group, many thanks for the unforgettable lunch breaks

and socials. Amanda, my running buddy and close friend- I had never found a same pacer, I will be back next week. Efstathia and Maria-Christina, my Greek besties, have long now proved that distance does not matter-it feels the same every time we meet in any part of this world. Many thanks to Evi, my flatmate, who has contributed to a wonderful home environment with humour, laughter and interesting storytelling.

Special thanks to my family, my parents and my siblings, Vily and Loukas, who have always been very supportive and encouraging in all my steps. I am grateful to Alex, for his continued support, understanding, patience and advice throughout these years. I had heard all about you, until I met you-I am glad we synchronised in this journey and I cannot wait for our new steps and challenges.

Last but not least, I dedicate this research to the loving memory of my grandparents Vasiliki Papagerogiou and Dimitris Diamantis who left us during this PhD.

Table of Contents

Chapter 1. General Introduction **1**

Chapter 2. Literature Review **7**

2.1. Introduction of the literature review 8

2.2. Bone structure, function and development 8

2.3. Bone Cells 10

2.3.1. Osteoblasts 10

2.3.2. Osteoclasts 11

2.3.3. Osteocytes 12

2.4. Bone Remodelling 12

2.5 Assessment of Bone Health 15

2.5.1. DXA 16

2.5.2. QCT, pQCT and HR-pQCT 17

2.5.3. MRI 18

2.5.4. BTMs 19

2.6. Key Determinants of Bone Health 27

2.6.1. Sex steroids and bone 27

2.6.2. Nutrition and bone 35

2.6.3. Mechanical loading and bone 39

2.7. Bone disorders 40

2.7.1. Osteoporosis 40

2.7.2. Stress fracture injury 41

2.8. EA 44

2.9. Aetiology of low EA 45

2.10. EA as part of the Female Athlete Triad and the RED-S models 46

2.11. Effects of energy restriction/low EA on bone metabolism and health in young, non-obese populations 50

2.11.1. Evidence from animal studies 50

2.11.2. Evidence from human studies -the Female Athlete Triad and the RED-S models 53

2.11.3. Interventional studies in humans 57

2.12. Endocrine alterations in response to low EA and bone metabolsim and health 62

2.12.1. Insulin 62

2.12.2. Cortisol 62

2.12.3. GH/IGF-1 63

2.12.4. Leptin 63

2.12.4. T_3 64
2.12.5. Ghrelin 65
2.12.6. PYY 65
2.12.7. GLP-1 and GLP-2 65
2.13. Summary 66
Chapter 3. General Methods 67
3.1. Ethical Approval 68
3.2. Recruitment 68
3.3. Questionnaires 68
3.3.1. Health Screen 68
3.3.2. Reproductive function 69
3.3.3. SCOFF questionnaire and weight history 69
3.3.4. Physical activity questionnaire 70
3.4. Anthropometric measurements 71
3.4.1. Height 71
3.4.2. Body mass 71
3.4.3. BMI 71
3.5. DXA 71
3.5.1. Quality control assurance 71
3.5.2. Total body scan measurement 71
3.6. Aerobic fitness test 73
3.7. Habitual dietary intake 74
3.7.1. Three-day food records 74
3.7.2. Dietary analysis 75
3.8. Measurement and estimations of EE 75
3.8.1. Measurement of lifestyle EE-Accelerometers 75
3.8.2. Measuring EEE 76
3.8.3. Day to day variability in EEE in physically active individuals by indirect calorimetry 76
3.9. EA 80
3.10. Variables of EE 81
3.11. Primary and secondary outcomes 81
3.12. AUC 82
3.13. Blood sampling and storage 82
3.14. Biochemical analysis 83

Chapter 4. Bone metabolic response to short-term low energy availability in physically active women 84
4.1. Introduction 85
4.2. Methods 87
4.2.1. Participants 87
4.2.2. Experimental design 87
4.2.3. Experimental procedures 88
4.2.4. Storage and analyses of blood samples 91
4.2.5. Biochemical analysis 91
4.2.6. Statistical analysis 91
4.3. Results 92
4.3.1. Participant characteristics 92
4.3.2. Baseline biochemistry 92
4.3.3. Baseline energy status and reproductive function 93
4.3.4. Compliance 94
4.3.5. Body mass 95
4.3.6. BTMs 95
4.3.7. Markers of calcium metabolism 98
4.3.8. Regulatory hormones 99
4.3.9. Reproductive hormones 100
4.3.10. Individual analysis 101
4.4. Discussion 103
Chapter 5. Bone metabolic response to short-term low energy availability in physically active men 106
5.1. Introduction 107
5.2. Methods 109
5.2.1. Participants 109
5.2.2. Experimental Design 109
5.2.3 Experimental Procedures 109
5.2.4. Storage and analyses of blood samples 110
5.2.5. Biochemical analysis 110
5.2.6 Statistical analysis 110
5.3. Results 111
5.3.1. Participant characteristics 111
5.3.2. Baseline biochemistry 111
5.3.3. Baseline energy status 112

5.3.4. Compliance 113
5.3.5. Body mass 113
5.3.6. BTMs 113
5.3.7. Markers of calcium metabolism 117
5.3.8. Regulatory hormones 118
5.3.9. Individual analysis 119
5.4. Discussion 121
Chapter 6. Bone metabolic response to short-term low energy availability in physically active men and women: a sex comparison 125
6.1. Introduction 126
6.2. Methods 128
6.2.1. Participants 128
6.2.2. Experimental design 128
6.2.3. Experimental procedures 128
6.2.4. Storage and analyses of blood samples 128
6.2.5. Biochemical analysis 128
6.2.6. Statistical analysis 128
6.3.Results 130
6.3.1. Participant characteristics 130
6.3.2. Baseline biochemistry 131
6.3.3. BTMs 132
6.3.4. Markers of calcium metabolism 136
6.3.5. Regulatory hormones 137
6.3.7. Individual analysis 143
6.4. Discussion 144
Chapter 7. Bone metabolic response to short-term low energy availability achieved by diet or exercise individually in physically active eumenorrheic women 149
7.1. Introduction 150
7.2. Methods 153
7.2.1. Participants 153
7.2.2. Experimental design 153
7.2.3. Experimental procedures 154
7.2.4. Storage and analyses of blood samples 156
7.2.5. Biochemical analysis 157
7.2.6. Measures of cognitive function and muscle function 157
7.2.7. Statistical analysis 157

7.3. Results 159
7.3.1. Participant characteristics 159
7.3.2. Baseline biochemistry 159
7.3.3. Baseline energy status and reproductive function 160
7.3.4. Body mass 161
7.3.5. BTMs 161
7.3.6. Markers of calcium metabolism 165
7.3.7. Regulatory hormones 167
7.3.8. Reproductive hormones 168
7.3.9. Individual analysis 168
7.4. Discussion 170
Chapter 8. Bone metabolic response to short-term low energy availability achieved by diet or exercise individually in combined oral contraceptive users 174
8.1. Introduction 175
8.2. Methods 177
8.2.1. Participants 177
8.2.2. Experimental design 177
8.2.3 Experimental procedures 177
8.2.4. Blood samples 177
8.2.5. Biochemical analysis 178
8.2.6. Statistical analysis 178
8.3. Results 179
8.3.1. Participant characteristics 179
8.3.2. Baseline characteristics 180
8.3.3. BTMs 180
8.3.4. Markers of calcium metabolism 184
8.3.6. Regulatory hormones 188
8.3.7. Reproductive hormones 190
8.3.7. Individual analysis 193
8.4. Discussion 194
Chapter 9. General discussion 199
9.1. Overview of key findings 200
9.1.1. Bone metabolic response to short-term low EA achieved through diet and exercise in physically active, eumenorrheic women 200
9.1.2. Bone metabolic response to short-term low EA achieved through diet and exercise in physically active men 201

9.1.3. A sex comparison: Bone metabolic response to short-term low EA in physically active women and men 202

9.1.4. Bone metabolic response to short-term low EA achieved by diet or exercise independently in physically active, eumenorrheic women 203

9.1.5. Bone metabolic response to short-term low EA achieved by diet or exercise independently in physically active combined OCP users 204

9.2. Programme impact and conclusions 205

9.3. Limitations 209

9.4. Future Work 211

Chapter 10. References 214

Chapter 11- Appendices 253

Chapter 1. General Introduction

Bone is a complex, multifunctional tissue that undergoes continuous renewal. The cell populations that accomplish this process, termed bone remodelling, are the osteocytes osteoclasts and osteoblasts that together, are organised in transient bone structures, named basic multicellular units (BMUs). Bone remodelling and structure (*i.e.,* mass, architecture- please also see section 2.5.) are critically dependent on processing of mechanical input and physiological cues from the systemic environment, and the conversion of these signals into the cellular events of bone remodelling. Remodelling is activated when osteoclasts are recruited to bone surfaces where they break down bone (bone resorption). Osteoblasts are sequentially attracted to the resorption sites where they refill empty cavities with bone that undergoes mineralisation (bone formation). The cellular link between bone formation and resorption is coupling, which maintains bone structure and strength in healthy bone (Proff & Romer, 2009; Crockett et al., 2011). Conversely, distortion of the balance between bone formation and resorption, favouring resorption, results in the development of micro-damage, which can eventually contribute to bone injury and disease such as stress fracture injury (Warden et al., 2006; 2014) and osteoporosis (Burghardt et al., 2010; Feng & MacDonald, 2011). Mechanical loading is a strong regulator of bone remodelling, which optimises bone mass, architecture and strength at sites imposed to stress (Wolff, 1892; Frost, 1987; Bonnet & Ferrari, 2010). In parallel, bone has non-mechanical functions; it is regulated by and regulates energy metabolism and distribution by communicating its energetic needs (Lombardi et al., 2015a; Karsenty and Ferron, 2012) and it has a key role in mineral homeostasis (Kular et al., 2012). Given these multiple functions of bone, it is not surprising that depending on regulatory inputs, optimisation or maintenance of bone structure through bone remodelling could presumably be compromised in favour of others (Turner 2001a; Iwaniec & Turner, 2016).

In humans, exercise (especially weight bearing activities) is mainly anabolic for bone, with athletes having higher bone mineral density (BMD), favourable adaptations to bone architecture particularly at weight bearing sites and greater bone strength than their sedentary counterparts (Scofield & Hecht, 2012; Tenforde & Fredericson, 2011; Ackerman et al., 2011; 2012a; Greene et al., 2012; Schipilow et al., 2013). However, when exercise is accompanied by inadequate dietary energy intake (DEI), this may compromise bone metabolism and health (Ihle & Loucks, 2004; Nattiv et al., 2007; De Souza et al., 2014a). Energy availability (EA), defined as DEI minus exercise energy expenditure (EEE) adjusted for lean body mass (LBM) or fat-free mass (FFM), represents the amount of energy that can be used for physiological functions after considering the energetic demands of exercise (Loucks & Thuma, 2003; Ihle & Loucks, 2004). Reduced EA results in physiological adaptations to spare energy, with priority given to the functions that are essential for

survival, including cellular maintenance, thermoregulation and sensory function (Wade et al., 1996; Wade & Jones, 2004), whereas less essential functions such as growth, reproductive function and bone health can be compromised (Loucks, 2007; 2013). The health consequences arising from energy deficiency are well identified in physically active females and are summarised as the Female Athlete Triad. This syndrome describes the complex interplay between low EA, menstrual dysfunction, and poor bone health (Nattiv et al., 2007; De Souza et al., 2014a). Female athletes with low EA and/or menstrual dysfunction are likely to develop low BMD and alterations in microarchitecture (Nattiv et al., 2007 Ackerman et al., 2011; 2012a), which may increase the risk of fracture in the short-term (*i.e.*, stress fracture injury) (Warden et al., 2014) and in the long-term (*i.e.*, osteoporotic fracture) (Loucks, 2007). Notably, low EA has a direct impact on bone metabolism and health, but also exerts indirect effects through the suppression of menstrual function (Nattiv et al., 2007; De Souza et al., 2005; 2008; Mallinson & De Souza, 2014).

The concept of Relative Energy Deficiency in Sports (RED-S) was introduced by the International Olympic Committee and expanded the definition of the Female Athlete Triad adding male athletes to the susceptible populations that may attain low EA and experience negative health (*i.e.,* bone and reproductive health) and performance consequences (Mountjoy et al., 2014; 2015). This change in terminology has been controversial in part due to the paucity of studies in men (De Souza et al., 2014b). Thus, further research into the effects of low EA on bone metabolism and health in men are necessary to provide an evidence base to determine if physically active men are affected to a similar extent by low EA, as their female counterparts.

Another overlooked sub-population in the Female Athlete Triad is physically active women using oral contraceptive pills (OCPs). Current evidence suggests that OCP use is highly prevalent among physically active females and there are at least an equal number of OCP users and non-users (Bennell et al., 1999a; Burrows & Peters, 2007). Combined OCP users present a different hormonal milieu than their eumenorrheic counterparts (Wiegratz et al., 2003; Burrows & Peters, 2007; Hansen et al., 2009; Blackmore et al., 2011), primarily due to down-regulation of endogenous oestrogen and progesterone as a result of the exogenous administration of these hormones (Burrows & Peters, 2007). The bone health of physically active OCP users is an issue of concern due to evidence suggesting that i) low oestrogen levels achieved by OCP may compromise peak bone mass in adolescent and young adult women (Polatti et al., 1995; Cibula et al., 2012) and ii) a combination of exercise and inadequate sex hormone levels may result in bone loss (Burr et al., 2000; Weaver et al.,

2001; Hartard et al., 1997). An important unanswered question that warrants investigation is how bone metabolism and structure respond to low EA in OCP users.

Stress fracture injuries are clinical manifestations of impaired bone health among physically active populations, including athletes and military recruits (Bennell et al., 1996a; Beck et al., 2000; Wentz et al., 2011; Warden et al., 2006; 2014). The exact pathophysiology of stress fracture injuries remains unknown and current models are theoretical, however, there is increasing agreement that stress fracture injuries are likely to result from disturbances in the homeostasis between micro-damage development and its repair. Factors associated with low EA have been implicated in the pathophysiology of stress fractures and conversely, stress fractures have been identified as a component of the Female Athlete Triad despite their exclusion in the diagram of the Female Athlete Triad (De Souza et al., 2014b). Low EA has been associated with low BMD (Nattiv et al., 2007) and alterations in micro-architectural properties of the bone (Ackerman et al., 2011; 2012a) that in turn, may modify the resistance of an applied load to the bone (Warden et al., 2014). Additionally, low EA may reduce the rate that microdamages are repaired (Ihle & Loucks, 2004; De Souza et al., 2008), leading to their accumulation and also affecting the ability of bone to resist loading.

Although there is evidence about long-term effects of EA on bone health, little is known about the sequence of events through which EA leads to these bone outcomes. Bone metabolic activity can be assessed indirectly by determining the levels of bone turnover markers (BTMs) to provide useful information in short-term dietary and exercise interventions (Grinspoon et al., 1995; Zanker & Swaine, 2000; Ihle & Loucks, 2004) before the detection of established changes in bone structure (Villareal et al., 2006; 2016). Ihle and Loucks (2004) demonstrated a dose-response relationship between EA (both diet and exercise manipulations) and BTMs in sedentary women and identified a threshold EA of 30 kcal·kg $LBM^{-1}\cdot d^{-1}$, below which there is a negative impact on BTMs. However, these data come from sedentary women, which may not be indicative of BTM responses to low EA in physically active women. As such, it is important to investigate the effects of low EA in this population. Understanding the mechanisms linking bone and low EA is of importance and has recently progressed. Oestrogen-independent mechanisms mediate the direct effects of EA on bone and include short-term endocrine responses to feeding/fasting (Walsh & Henriksen, 2010), and longer-term endocrine changes to energy stores in order to save energy for vital functions and maintain life (Miller, 2011; Warren, 2011; Misra & Klibanski, 2011; 2014). Oestrogen-dependent mechanisms communicate the indirect effects of EA on bone though menstrual dysfunction. Amenorrheic athletes present with oestrogen deficiency and are at higher risk for low BMD (Zanker & Swaine, 1998b; Christo et al., 2008) and

stress fracture injuries compared with eumenorrheic athletes (Nattiv et al., 2013; Bennell et al., 1999b). These issues require further investigation in order to reduce possible adverse bone effects and promote strategies that favour beneficial endocrine responses. Understanding the endocrine factors that regulate bone metabolism in response to low EA and the differences in endocrine responses, when low EA is diet- or exercise-induced would help minimise adverse bone outcomes.

Our understanding of the effects of short-term low EA on bone metabolism in men is limited. Only Zanker & Swaine (2000) have examined the effects of exercise and/or reduced food intake on bone metabolism in trained men, showing a reduction in bone formation without an effect on bone resorption. However, energy restriction was determined using energy balance rather than EA (Zanker & Swaine, 2000) defined as total energy expenditure (TEE) minus DEI. As such, the determination of energy balance requires the measurement of TEE which comprises resting metabolic rate (RMR), thermic effect of food and exercise energy expenditure (EEE)], whereas only the measurement of EEE is essential for determining EA. A number of characteristics, such as body composition, regulatory and reproductive hormones differ between men and women (De Souza et al., 2014b; Williams et al.,2015; Roberts et al., 2016) and may contribute to differences in BTM responses (Ihle & Loucks, 2004; Zanker & Swaine, 2000; Shapse & Sukumar, 2012). Therefore, the effects of EA on bone metabolism in men await investigation and a comparison of these responses with those in women would be of particular interest for clarifying the interaction between EA and bone health proposed in the Female Athlete Triad (Nattiv et al., 2007; De Souza et al., 2014a) and the RED-S models (Mountjoy et al., 2014; 2015). Similarly, it is unknown if the responses of bone metabolism to low EA in eumenorrheic women may be similar or different from those in OCP users. Thus, the investigation of the effects of low EA in these groups will inform our understanding of OCP use among physically active women.

Another fundamental gap in the existing knowledge base in this area is whether EA restricted by diet or exercise alone has the same or different effects on BTM responses. Current studies have examined dietary restrictions (Grinspoon et al., 1995) or diet and exercise manipulations (Zanker & Swaine, 2000; Ihle & Loucks, 2004) on BTMs, but no study yet has explored the same level of reduced EA by diet or exercise independently, a critical question ahead of determining prevention and treatment strategies.

The effects of low EA on bone metabolism warrant investigation. There is some evidence for unfavourable effects of low EA in sedentary women following three distinct levels of reduced EA (10, 20 and 30 kcal·kg $LBM^{-1}\cdot d^{-1}$) on BTMs, but the effects of intermediate

levels of energy restriction *i.e.*, 20-30 or 10-20 kcal·kg $LBM^{-1}·d^{-1}$ have not yet been explored. Understanding the effects of EA between 10 and 20 kcal·kg $LBM^{-1}·d^{-1}$ on bone health is particularly important, since EA of approximately 16 kcal·kg $LBM^{-1}·d^{-1}$ occur in some amenorrheic athletes (Thong et al., 2000). The influence of short-term reduced EA on bone metabolism in physically active populations and differences in bone metabolism between men and women or between eumenorrheic women and women using OCPs, have not been previously described. Low EA can develop from inadequate DEI, high EEE or a combination of the two, but it remains unexplored if diet- or exercise-induced low EA has the same or different effects on BTMs.

The overarching aim of the programme of work was to investigate the effects of short-term EA on bone metabolism and health. Some of the fundamental gaps in the literature highlighted above were addressed:

1. Studies 1 and 2 explored the effects of short-term low EA achieved by diet and exercise on BTMs in physically active eumeorrheic women (reported in Chapter 4) and men (reported in Chapter 5).
2. The data collected in Studies 1 and 2 were combined to directly compare the BTM responses to short-term low EA between physically active women and men (reported in Chapter 6).
3. Studies 3 and 4 investigated the effects of short-term low EA on BTMs dependent on whether EA is diet- or exercise-induced in physically active, eumenorrheic women (reported in Chapter 7) and women using combined OCPs (reported in Chapter 8).
4. The data collected in Studies 3 and 4 were combined to directly compare the BTM responses to short-term low EA between physically active, eumeorrheic women and combined OCP users (reported in Chapter 8).
5. Studies 1-4 provided information on the changes in regulatory and reproductive hormones and markers of calcium metabolism underpinning the effects of low EA on BTMs (reported in Chapters 4-8).

Chapter 2. Literature Review

2.1. Introduction of the literature review

Chapter 2. provides a review of the existing literature on bone health and EA. The first part of this review focuses on bone biology and physiology to provide the necessary background for understanding bone health. The main characteristics, advantages and limitations of current methods to assess bone health are reviewed; with emphasis placed upon BTMs that were used to assess bone health in the current programme. Several factors underpin bone health such as genetics, sex hormones, nutritional factors and mechanical loading. In the context of this PhD, there will be only a light touch on genetics, since the focus will be placed upon sex hormones, nutrition and mechanical loading due to their relevance with the components and/or the study populations of the interventions in this programme. Osteoporosis and stress fracture injury will be discussed to provide the basis of long- and short-term clinical manifestations of poor bone health and provide some context for the potential implications of this work.

The second part of the literature review will provide a review of the concept of EA in exercise physiology and the role of low EA as the cornerstone component of the Female Athlete Triad (Nattiv et al., 2007; De Souza et al., 2014a) and the RED-S models (Mountjoy et al., 2014; 2015). Despite the rapidly growing body of literature on low EA, our understanding of the potential effects of EA on bone metabolism and health remains incomplete. Current evidence of the effects of low EA on bone metabolism and health from animal models; from research in the area of the Female Athlete Triad and the RED-S models and from interventional studies in humans will be reviewed and the knowledge gaps will be identified. Regulatory hormones with well-established or emerging bone actions may change in response to energy deficiency; thus, the last part of this review will review these changes, while highlighting the need for future research to assess regulatory hormone responses to low EA in relation to bone-related outcomes in relevant populations.

2.2. Bone structure, function and development

Bone is a dynamic multifunctional tissue. The axial and appendicular skeleton facilitate a number of mechanical functions, including provision of attachment sites for ligaments, tendons and muscles to allow locomotion; protection of internal organs (*i.e.*, ribs, vertebrae and skull), application of mechanical forces (*i.e.*, fingers) and resistance to mechanical stresses (*i.e.*, bones at weight bearing sites) (Morgan et al., 2013). Bone can be separated into cortical and trabecular compartments. Cortical bone contributes approximately 80% of adult skeleton and is primarily found on the diaphysis (long shaft) of long bones and bone surfaces. Cortical bone lies between surfaces or bone envelopes; the outer envelope, the

periosteum faces the soft tissue, whereas the inner envelope, the endosteum, wraps the inner trabeculae. Owning to its high level of calcification, cortical bone is dense and strong, thus, its main role is structure and protection. Trabecular bone is primarily found in the axial skeleton and the epiphyses (ends) of long bones. It is light due to its porous build-up to allow energy absorption and has a greater metabolic activity compared to the cortical bone (Morgan et al., 2013). The basic characteristics of bone structure are presented in Figure 2.1.

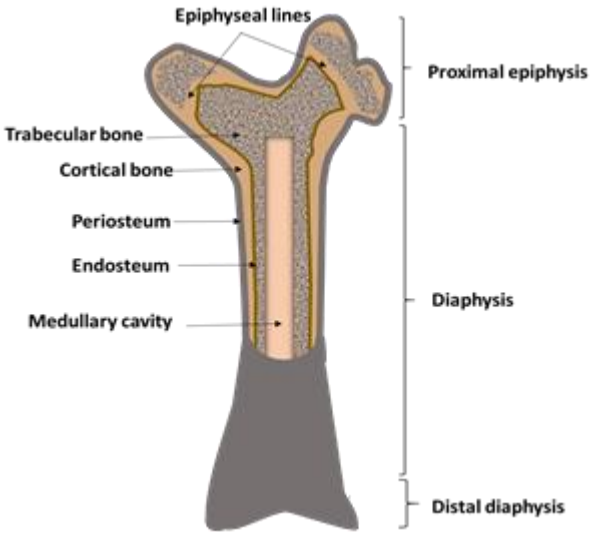


Figure 2.1. Basic structure of bone (adapted from St-Arnaud, 2008).

In addition to its mechanical functions, bone is an important endocrine organ that is functionally linked to other metabolically active tissues via endocrine factors. Bone is involved in the homeostasis of calcium and phosphorus, which is controlled by the calciotropic hormones (Burr, 2002; Karsenty and Ferron, 2012). Bone also plays an integral role in energy homeostasis and reproduction (Wee et al., 2016; Lombardi et al., 2015a; Karsenty & Ferron, 2012). Bone regulates energy metabolism and distribution by communicating its energetic needs through the production of molecules [*e.g.*, osteocalcin (OC)); which, in turn, act on other tissues and organs such as muscle, adipose tissue, liver, pancreas and gonads (Lombardi et al., 2015a; Karsenty and Ferron, 2012). These organs secrete several regulatory hormones (*e.g.*, insulin, leptin) that are involved in the regulation of bone homeostasis in response to EA (Lombardi et al., 2015a; Karsenty and Ferron, 2012).

Bone is in a dynamic state that enables its modelling during bone development and growth, and remodelling during adult life (Morgan et al., 2013). Bone remodelling allows for adaptations to its surrounding environment, replacement of damaged tissue and, as mentioned earlier, the tight control of mineral concentrations in the blood (Burr, 2002; Proff

& Romer, 2009). In the remodelling process, the old or damaged bone is removed, and sequentially replaced with new bone of analogous volume (Martin & Seeman, 2008; Proff & Romer, 2009). Alterations in bone modelling and predominantly remodelling determine the structural and material properties of the bone throughout the lifespan (Bouxsein, 2005; Seeman & Delmas, 2006). The attainment of bone mass occurs mostly during childhood and adolescence, when bone increases both in length and width (MacDonald et al., 2013; Khosla & Pacifici, 2013). Linear bone growth refers to the ossification of growth plates until they fuse and radial bone growth is characterised by periosteal apposition, and persists even after puberty (MacDonald et al., 2013; Khosla & Pacifici, 2013). Greater periosteal apposition in boys than in girls, together with the longer period of bone acquisition in boys (about 1.5 years), account mostly for the sexual dimorphism of adult bone (Khosla & Pacifici, 2013; McDonald et al., 2013; Bailey et al., 1999). Notably, there is no agreement about the exact age of peak bone mass acquisition, which ranges from the second to the fourth decade (Baxter-Jones et al., 2011; Recker et al., 1992; Berger et al., 2010). Some of these discrepancies may be explained by different study designs (cross-sectional, longitudinal) and inadequate control of biological age or body composition. Following the attainment of peak bone mass, bone is lost at a slow, steady rate as part of the ageing process, with accelerated bone loss seen in women in the peri-and early post-menopausal years (Clarke & Khosla, 2012). Importantly, the rate and magnitude of bone loss varies considerably amongst individuals (Cauley et al., 2009) and depends on a complex interplay between genetics, endocrine profile and lifestyle factors (*i.e.,* nutrition and exercise). By favourably modifying the factors that influence bone health, optimisation of peak bone mass achieved in young adulthood and attenuation of bone loss later in life can reduce the risk for fracture (Boreham & McCay, 2011; MacDonald et al., 2013; Weaver et al., 2016).

2.3. Bone Cells

2.3.1. Osteoblasts

Osteoblasts originate from mesenchymal stem cells available in the bone marrow and their main role is bone formation (Figure 2.2.). Other cell lineages differentiating from the same precursors are chondrocytes, adipocytes and myocytes, which share the same characteristics (Bianco et al., 2010). Osteoblast differentiation is promoted by the canonical wingless (Wnt)-β-catenin pathway, bone morphogenic proteins (BMPs) and master transcriptional factors such as Runt-related transcription factor 2 (Runx2) and Osterix (Osx) (Nakashima et al., 2002; Crockett et al., 2011). The canonical Wnt-β-catenin pathway is of critical importance for osteoblast differentiation, but also for the activity of mature osteoblasts. Wnt proteins bind to a low-density lipoprotein receptor-related protein 5 and 6 (LRP5/6) co-

receptor to disrupt a protein complex made of adenomatous polyposis coli, axin and glycogen synthase kinase 3β (GSK-3β). The suppression of GSK-3β inhibits the phosphorylation of ß-catenin, resulting in the accumulation of ß-catenin in the cytoplasm and its translocation to the nucleus, where ß-catenin contributes to the transcription of target genes. Several antagonist molecules regulate the canonical Wnt-β-catenin pathway. Among them, Dickkopf 1 (DKK-1) secreted by osteocytes, binds to LRP5/6 to form a complex that result in the removal of LRP5/6 from the cell surface (Proff & Romer, 2009). Sclerostin, a product of the sclerostin encoding gene (SOST) also produced by osteocytes, binds to the LRP5/6 receptor to prevent the binding of Wnt protein (Proff & Romer, 2009). Osteoblasts are the principal cells for bone formation and mineralisation that synthesise and release collagen and other non-collagenous constitutes to form bone matrix and express genes necessary for these procedures. Osteoblasts express a number of membrane proteins (*i.e.*, bone specific alkaline phosphatase-BALP, OC) and receptors for primary bone regulating hormones (*i.e.*, parathyroid hormone-PTH and 1,25-dihydroxyvitamin D (1,25-$(OH)_2$D)) (Proff & Romer, 2009; Crockett et al., 2011). In addition to their role in bone formation, osteoblasts are important regulators of osteoclastogenesis and osteoclast activity through the production of the ligand for receptor activator of nuclear factor kappa B (RANKL) and osteoprotegerin (for more details, please see 2.3.2.) (Proff & Romer, 2009). Osteoblasts can i) become embedded into the bone matrix and differentiate into osteocytes, ii) remain in bone surfaces as bone- lining cells, or iii) undergo apoptosis (Manolagas et al., 2002; Crockett et al., 2011) (Figure 2.2.).

2.3.2. Osteoclasts

Osteoclasts are multinucleated, giant cells located on bone surfaces that facilitate bone resorption (Figure 2.2.). They originate from cells of the monocyte-phagocyte lineage and important factors for osteoclast differentiation, activity and survival are the macrophage colony-stimulating factor (M-CSF), RANKL and OPG (Proff & Romer, 2009; Crockett et al., 2011). M-CSF binds to its receptor (colony stimulating factor 1 receptor (c-fms)) and stimulates proliferation and differentiation of osteoclast progenitors (Crockett et al., 2011). It also reinforces osteoclast activity and inhibits osteoclast apoptosis (Crockett et al., 2011). RANKL is a membrane bound on the osteoblast surface and thus, its binding to the receptor activator of nuclear factor kappa B (RANK), which is present on the surface of osteoclast, allows cell to cell interaction between osteoblasts and osteoclasts, which, in turn, promotes osteoclast differentiation. In addition to RANK, RANKL can bind to its decoy receptor OPG to inhibit osteoblast differentiation. Loss of function mutations of RANKL/RANK suppress completely osteoclastogenesis, resulting in a high bone mass phenotype named osteopetrosis

(Guerrini et al., 2008). Once differentiated, osteoclasts are attracted to bone sites requiring resorption and attach to bone surfaces, where the cell membrane is folded, and therefore, appears as a ruffled border (Boyce et al., 2009). In the areas that undergo bone resorption, osteoclasts produce acid and secrete enzymes (*e.g.,* cathepsin K, matrix metalloproteinases) that result in bone mineral dissolution and degradation of collagen fragments (Teitelbaum, 2007; Negishi-Koya et al., 2009).

2.3.3. Osteocytes

Osteocytes are the most abundant type of bone cells, composing over 90-95% of the total bone cell population. Osteocytes possess a number of dendrite-like cytoplasmic processes that allow communication with each other and with other cells including the lining cells on bone surfaces, endothelial cells and components of bone marrow (Bonewald et al., 2011). The cell bodies of osteocytes are engulfed within individual cavities in the bone matrix, termed lacunae, and the processes lie within canals, termed canaliculi (Manolagas & Parfit, 2010). Over the last few years, new evidence has supported the role and function of osteocytes and they are now thought to be the maestros of bone remodelling (Bonewald et al., 2011). Osteocytes have a critical role in the activation of bone remodelling, as they sense bone defects, mechanical loading or changes in metabolic signals and through signalling pathways control the migration of osteoclasts and osteoblasts to the places that need remodelling (Bonewald, 2011; Crockett et al., 2011). They also have important endocrine functions; they produce factors that affect the recruitment and differentiation of osteoblasts and osteoclasts [*e.g.,* RANKL and OPG, osteopontin, regulator of bone mineralisation), dentin matrix acidic phosphoprotein 1 (DMP-1, promoter of bone mineralisation), sclerostin and DKK-1 (antagonists of Wnt pathway and thus, inhibitor of bone formation)] (Bonewald et al., 2011; Manolagas & Parfit, 2010).

2.4. Bone remodelling

The cellular mechanisms that allow bone to adapt to prevailing conditions are modelling and remodelling**.** Bone modelling involves the formation of new bone without previous bone resorption. This process occurs during bone development and growth and results in alterations in bone size and shape (Seeman & Delmas, 2006; Baron & Kneissel, 2013). Bone modelling is persistent in adults in cases of fracture repair, development of malignant bone metaplasia and in the initial adaptation of bone to biomaterials (Chappard et al., 2008). During bone remodelling, old or damaged bone is resorbed first and subsequently, new bone is formed to replace it. Bone remodelling is a highly co-ordinated process carried out by the synchronised action of osteoblasts, osteoclasts and osteocytes within bone remodelling units

(Figure 2.2). Bone remodelling can occur in a random fashion in response to hormonal stimuli (*e.g.,* PTH) and is mainly responsible for calcium release into the circulation (stochastic bone remodelling) or it can be targeted to specific areas of bone loading or damage, which are presumably identified by osteocyte apoptosis (Warden et al., 2014; Burr, 2002). Bone remodelling takes place on all bone surfaces, but the rate of this process is greater in the trabecular, rather than in the cortical compartment, mainly due to the greater surface area per unit bone volume of trabecular bone (Morgan et al., 2013; Seeman & Delmas, 2006). Bone remodelling involves subsequent phases of activation, resorption, reversal and formation (Figure 2.2). Maintenance of structural, metabolic and biomechanical integrity together with the control of mineral homeostasis are all achieved through bone remodelling (Burr, 2002; Proff & Romer, 2009). Given the importance of these cellular activities, it is unsurprising that in adults, bone resorption and bone formation are closely coupled. In contrast, an imbalance between bone formation and resorption occurs in several bone-related conditions and results in bone loss. For example, osteoporosis is a common bone disease resulting from oestrogen deficiency (*e.g.,* postmenopausal osteoporosis), immobilisation or drugs (*e.g.,* glucocorticoids), in which bone resorption is favoured and bone loss occurs (Feng & McDonald, 2011; Riggs et al., 1998).

It was long believed that bone remodelling was under the control of key circulating hormones including PTH, Vitamin D and sex hormones (McKenna and Frame, 1987). Advances over recent decades have shown that the regulation of bone remodelling is more complex and, in addition to these hormones, is controlled by central, systemic and local factors at different stages of the bone remodelling cycle including differentiation and activity of osteoblasts and osteoclasts and intracellular communication (Crockett et al., 2011). For example, PTH, interleukin-1 (IL-1) and 6 (IL-6), tumour necrosis factor α (TNF-α), prostaglandin E2 and $1,25(OH)_2D$ stimulate the expression of RANKL and promote osteoclast differentiation and bone resorption (Proff & Romer, 2009). Growth hormone, PTH, transforming growth factor *β* (TGF-*β*), insulin growth factor 1 (IGF-1) and 2 (IGF-2) upregulate the transcriptional factors necessary for osteoblastogenesis and bone formation (Glass & Karsenty, 2006). Osteoblast function is also regulated by the sympathetic nervous system; specifically, stimulation of *β2*-adrenergic receptors, present on osteoblasts, has a suppressive effect on osteoblastic activity, whilst reinforcing the function of osteoclasts (Takeda et al., 2002).

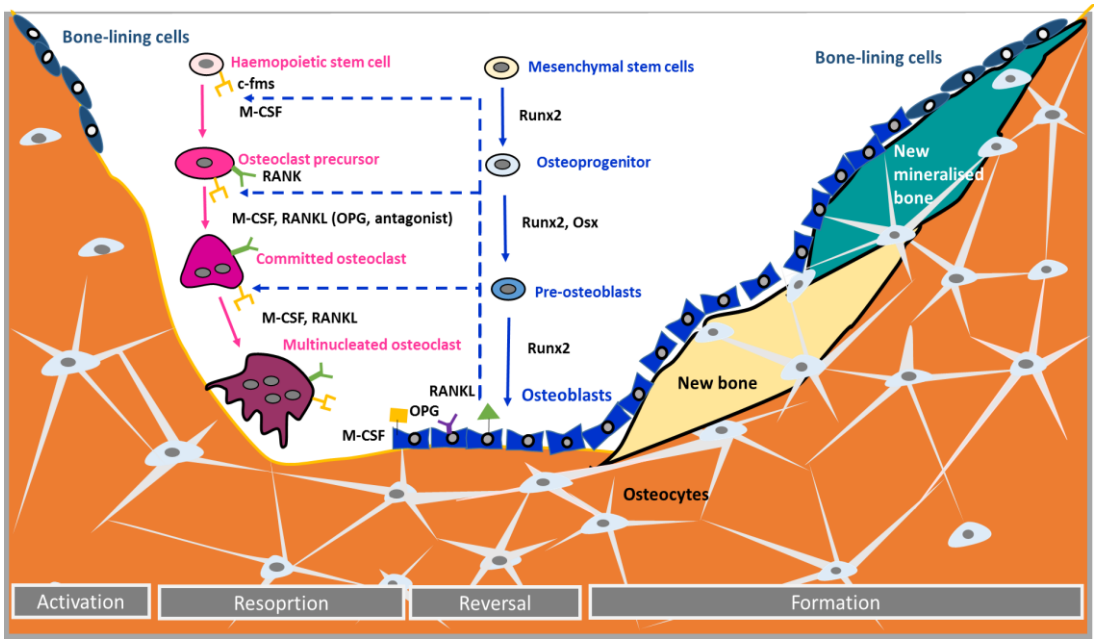


Figure 2.2. Overview of bone remodelling and differentiation pathways for osteoclasts and osteoblasts. Bone remodelling is activated in response to various stimuli (mechanical strain, damage, changes in hormonal factors or calcium levels). Osteocytes detect these changes and are stimulated or die with apoptosis initiating further signalling pathways. These, together with signals from osteoblast lineage and factors from the bone matrix, promote the recruitment and differentiation of osteoclasts from haemopoietic stem cells and subsequent osteoclast activity (bone resorption) and osteoclast survival. M-CSF and RANKL can bind to the receptors c-fms and RANK, which are located on the osteoclast precursor, and stimulate them to differentiate to fully activated osteoclasts and reinforce their activity and survival. OPG that is secreted by osteoblasts competes RANK for RANKL binding. Following bone degradation, osteoclasts undergo apoptosis and osteoblasts are recruited and differentiated from mesenchymal stem cells with Runx2 and Osx being key transcriptional factors in these processes (reversal phase). During the formation phase, osteoblasts deposit new bone material, which becomes mineralised. Some osteoblasts are entrapped inside the bone matrix and differentiate into osteocytes, some undergo apoptosis and some others cover the new bone surface as bone lining cells. RANK: receptor activator of nuclear factor kappa B; RANKL: receptor activator of nuclear factor kappa B ligand; Runx2: Runt-related transcription factor; Osx: osterix; OPG: osteoprotegerin; c-fms: colony stimulating factor 1 receptor; M-CSF: macrophage colony-stimulating factor. Adapted from Proff & Romer, 2009; Bassett & Williams, 2008 and Nappi et al., 2012.

2.5 Assessment of bone health

Bone strength, the load-bearing capacity of bone prior to fracture occurrence (Bouxsein, 2005; Manske et al., 2010), has increasingly gained scientific and clinical interest (Davison et al., 2006; Seeman & Delmas, 2006; Bouxsein & Seeman, 2009). It is determined by parameters of bone structure (*e.g.*, bone mass, shape and microarchitecture) and material properties of the bone matrix (*e.g.*, mineralisation, porosity, collagen characteristics, microdamage accumulation) (Davison et al., 2006; Bouxsein, 2005) (Figure 2.3.). Importantly, bone remodelling mediates the changes in all of these properties by altering the balance between bone formation and bone resorption (Figure 2.3.).

There are a number of ways to assess bone strength and its determinants. Bone mass, expressed as bone mineral content (BMC) and BMD, is the most widely measured bone parameter and can be determined by dual-energy X-ray absorptiometry (DXA). Bone geometry can be assessed using quantitative computed tomography (QCT). Recent developments of QCT; namely peripheral quantitative computed tomography (pQCT) and high-resolution quantitative peripheral computed tomography (HR-pQCT), are useful tools to assess bone geometry, but also microarchitecture. Magnetic resonance imaging (MRI) is also used to assess bone structure indirectly and for diagnosing stress fracture injuries. Similar to QCT developments, MRI advances such as high-resolution MRI (HR-MRI) have been used to predict parameters of bone microarchitecture (Link et al., 2003). In addition to these measures of bone mass and structure, bone metabolic activity can be assessed indirectly by determining BTM levels (Figure 2.3.). Table 2.1 shows the advantages and disadvantages of each method. The techniques used to measure the material properties of the bone matrix are either invasive (*e.g.*, bone biopsy) or still under development (*e.g.*, set-up of testing protocols and optimisation of testing parameters for bone microindentation) (Diez-Perez et al., 2010) and, as such, have limited applicability in clinical settings so far. Therefore, they will not be described in this overview.

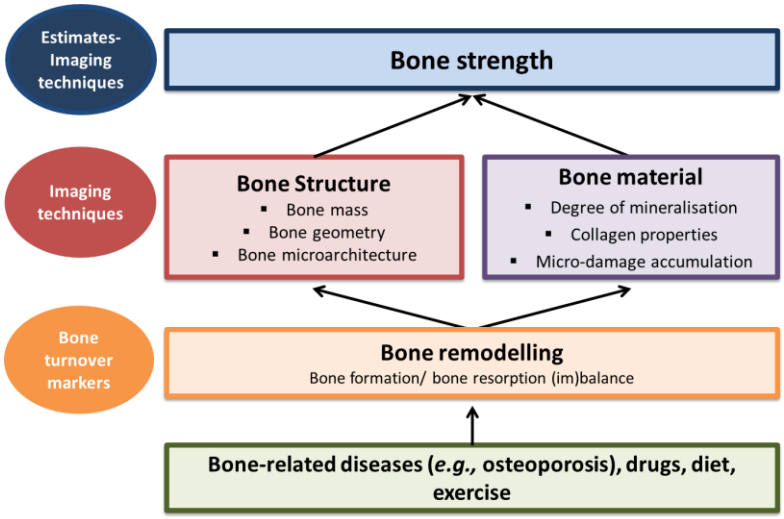


Figure 2.3. Determinants of bone strength and commonly used methods for their assessment. Adapted from Bouxsein (2005).

2.5.1. DXA

DXA is a quantitative measure of bone mass based on the different absorption rates between high and low-energy X-rays when these pass through the body. High density X-rays are able to penetrate the soft tissue and the bone, whilst low-density X-rays can only pass through soft tissue. Thus, the difference between the two provides a quantification of BMC (g) (Golden, 2015). BMC is divided by the scanned area to estimate areal BMD ($g \cdot cm^{-2}$); resulting BMD measure is areal rather than volumetric. As areal BMD strongly correlated with bone strength (r^2=50-90%) and is a predictor of fracture risk (Marshall et al., 1996), the current diagnostic criteria for osteoporosis by the World Health Organization is based on BMD measurement by DXA (Kanis, 1994.). DXA has several strengths; low radiation exposure, high precision and reproducibility (coefficient variation (CV): 0.6-1.9%; Shepherd et al., 2006), relatively low cost, when the equipment is available, ease of use and brief examination times (Borer, 2005; Bouxsein & Seeman, 2009). When used in conjunction with hip structural analysis (applied to DXA images), DXA can provide estimations of parameters of bone geometry at the proximal femur such as cross-sectional area, section modulus (estimate of resistance to bending), and buckling ratio (estimate of resistance to compression) (Ackerman et al., 2013a). Hip structural analysis is, however, depends on technical expertise of scanner technologist and is limited by poor image quality and a number of assumptions (e.g., relevant with bone tissue mineralisation) (Beck et al., 2007).

Despite the aforementioned advantages, only two-dimensional measurements are obtained, which do not allow the detection of differences in areal BMD due to different bone sizes

(Carter et al., 1992). Areal BMD, determined by DXA, overestimates the true vBMD in larger bones, whereas it underestimates it in smaller bones (Duan et al., 1999). Another limitation of DXA is that it does not provide information about bone geometry and microarchitecture, which is important, as changes in BMD cannot be translated into morphological changes. For example, bone gain in response to a specific stimulus, such as mechanical loading, may occur in trabecular sites, cortical sites or in both, with these changes being indistinguishable with areal BMD measurements alone. Small alterations in bone dimensions, which may translate into substantial changes in bone strength, cannot be identified either (MacDonald et al., 2013; Manske et al., 2010; Golden, 2015).

2.5.2. QCT, pQCT and HR-pQCT

The most commonly measured variables of cortical bone are cortical area, cortical diameter, cortical thickness and cortical porosity (Manske et al., 2010; Turner et al., 2002; Davison et al., 2006). Increases in the outer diameter and in cortical thickness of a long bone and reductions in cortical porosity, increase bone strength (Turner et al., 2002; McDonald et al., 2013; Bouxsein, 2005). Trabecular microarchitecture is characterised by trabecular volume, the number of trabeculae (trabecular number) in a given volume (trabecular density), trabecular thickness, trabecular morphology (plate-like or rod-like), and variables of trabecular connectivity (*i.e.*, intertrabecular spacing and seperations) (Chappard et al., 2008; Bouxsein & Seeman, 2009; Davison et al., 2006).

QCT is a three-dimensional technology that measures vBMD ($g \cdot cm^{-3}$) and provides information about the cortical and trabecular contributions of bone (Manske et al., 2010). Reliability of trabecular BMD estimates using QCT ranges between 0.6 and 4% (Engelke et al., 2008). The more recent versions of QCT, pQCT and HR-pQCT, measure vBMD of the peripheral skeleton, such as the radius and tibia, therefore, utilising low radiation doses, similar to those used by DXA (<3 mSv) (Manske et al., 2010). Both pQCT and HR-pQCT distinguish cortical and trabecular bone, providing information about the pathophysiology of bone-related conditions and the effectiveness of interventions. pQCT can provide estimates of cortical thickness, as well as cortical and trabecular density from cross-sectional images. HR-pQCT is able to produce images of high resolution and therefore, allows evaluation of bone microarchitecture (*e.g.,* cortical thickness, trabecular number, thickness and separation). HR-pQCT measurements are highly reproducible with a CV of less than 1% for density and less than 4.5% for variables of microarchitecture (MacNeil et al., 2008). Finite element analysis is a modelling technique, which utilises QCT or HR-pQCT images, to provide estimates of bone strength parameters, such as stiffness and failure load (Bouxsein,

2005; Manske et al., 2010). These parameters have been associated with fractures and can improve their prediction (Boutroy et al., 2008). The application of individual trabecula segmentation analysis on HR-pQCT is a clinically sensitive technique that provides information about trabecular morphology (plate-like versus rod-like), orientation and connectivity (Mitchell et al., 2015). This piece of equipment is, however, not routinely available in clinical settings.

2.5.3. MRI

MRI utilises magnetic fields with specialised sequences of radiofrequency pulses to produce 3D images of bone structure (Wehrli, 2006). Bone structure is indirectly evaluated though measurements of bone marrow and soft tissue (Wehrli, 2006). The absence of radiation and the sensitivity to detect changes in bone marrow (Bouxsein & Seeman, 2009; Golden, 2015) are some of the strengths of this method. However, it is technically demanding and expensive and more time consuming than the other methods discussed; thus, it is used mostly in research. Its use in clinical settings for bone health assessment is limited, however, MRI scans are often requested to evaluate musculoskeletal injuries *(e.g.,* stress fracture injuries) (Warden et al., 2014). HR-MRI can be performed at peripheral skeletal sites (e.g., distal radius, distal tibia and calcaneus) using MRI scanners with specific coils to obtain parameters of bone microarchitecture. Due to limited resolution, this technique cannot provide accurate measures of bone microarchitecture; however, the estimation of bone microarchitectural properties based on HR-MRI images have been highly correlated with those determined with techniques of greater resolution (Link et al., 2003; Bouxsein & Seeman, 2009).

Table 2.1. Advantages and limitations of current imaging equipment for bone health assessment (Adapted from Manske et al., 2010; Bouxsein & Seeman, 2009; Golden, 2015).

	DXA	**QCT**	**pQCT**	**HR-pQCT**	**MRI HR-MRI**
Sites scanned	Lumbar spine Hip Distal radius Total body	Lumbar spine Hip Distal radius	Distal radius Distal tibia	Distal radius Distal tibia	Central and distal sites
Radiation exposure (µSv)	1-6	30-7,000	<3	<5	N/A
Scan duration	1-6 min	<1min	3 min	3min	10 min
Differentiation of cortical-trabecular bone	No	Yes	Yes	Yes	Yes
Bone microarchitecture	No	No	No	Yes	Yes
Reproducibility (%CV)	0.6-1.9%	0.6-4%	0.7-1.7%	1-4.5%	3-8%

DXA: Dual-energy X-ray absorptiometry QCT: quantitative computed tomography; pQCT peripheral quantitative computed tomography; HR-pQCT: high-resolution peripheral quantitative computed tomography; MRI: Magnetic resonance imaging; HR-MRI: high resolution MRI; CV: Coefficient Variation.

2.5.4. BTMs

BTMs are measures of the dynamics of bone metabolic activity and are typically categorised into markers of bone formation or bone resorption. Potential advantages of their use include the non-exposure to radiation, high sensitivity in detecting alteration in bone metabolism, ease of collection and analysis. Measurement of BTMs has relative low cost (*i.e.*, compared to the purchase of a DXA scanner) and requires blood or urine collection, which are tolerable and less onerous than a bone biopsy (Lombardi et al., 2012a; Banfi et al., 2010). Some BTMs can be measured in the urine due to their small size, whereas some larger sized molecules can be determined in blood. BTMs are analysed using radioimmunoassays, immunoradiometric assays, enzyme linked immunosorbent assays (ELISA) and chemiluminesence immunoassays (ECLIA) and are predominately automated procedures, which allow the convenient, high volume and more accurate measurement of BTMs in research and clinical settings (Hlaing & Compston. 2014). BTM are systematic in nature and thus, reflective of bone turnover of the whole skeleton and not specific to affected skeletal sites. The currently available BTMs are present in bone, but also in other non-skeletal tissues (*i.e.*, all markers deriving from Type 1 collagen predominantely originate from bone, but also to a small extent from skin, dentin, cornea, vessels or tendons), therefore, their levels may be less relevant to bone changes (Lombardi et al., 2012a; Wheater et al., 2013). BTMs are not always specific to bone formation or resorption exclusively, instead, they may reflect cumulative bone turnover (Banfi et al., 2010; Lombardi et al., 2012a). BTMs are influenced by analytical variability and biological variability (please see section 2.6.4.3). The type of sample, the duration and the temperature of storage before analysis vary widely and depend

predominantly on the equipment and kit used. Importantly, large variability has been associated with bone markers measured in the urine (15-25%) and serum (10-15% and 3-5% with automated assays), attributed to the intra- and inter-assay variability, but also to biological factors (Lombardi et al., 2012a; Vasikaran et al., 2011; Wheater et al., 2013).

In a clinical setting, BTMs are used to assess the effectiveness of osteoporosis treatments, evaluate patients' compliance to therapy, predict bone loss and risk of developing osteoporosis and identify individuals at high risk for sustaining osteoporotic fractures (Vasikaran et al., 2011; Wheater et al., 2013). The synchronous measurement of BTMs and BMD can provide a multidimensional approach to patient management (Dogan & Posaci, 2002). The use of BTMs for research purposes has increased rapidly over recent years, as they can capture early changes in bone metabolism as opposed to static measures that are only capable of revealing already established changes of altered bone metabolism. The assessment of multiple BTMs is recommended to evaluate changes in bone resorption, formation or total bone turnover and recently, BTMs have been used to assess bone responses to EA (Ihle & Loucks, 2004; Zanker & Swaine, 2000), exercise only (Scott et al., 2010; 2012; 2013), or caloric restriction only (Grinspoon et al., 1995).

2.5.4.1. Markers of bone formation

2.5.4.1.1. Carboxyl- or Amino-terminal Pro-peptides of Type 1 Procollagen (P1CP and P1NP)

The mature molecule of type I collagen is formed after the enzymatic cleavage of terminal peptides from the procollagen molecule; namely P1NP and P1CP. The release of P1CP and P1NP into the circulation is equimolar to newly synthesised type I collagen (Banfi et al., 2010; Lombardi et al., 2012a). Although type I collagen is the main component of bone, it is also present in skin, dentin, cornea, vessels and tendons (Banfi et al., 2010). P1NP may better reflect bone formation compared to P1CP, possibly due to differences in the regulation of their catabolism. The clearance of P1NP is achieved by scavenger cells at a steady pace, whilst P1CP is rapidly cleared by mannose receptor in hepatic endothelial cells, which are influenced by several hormones, especially thyroid hormones and IGF-1 (Hannon & Eastell, 2006; Luftner et al., 2005; Wheater et al., 2013). P1NP is released in trimeric form, but it undergoes rapid thermal degradation to form a monomeric form. P1NP can be analysed using two types of assays (RIA, ECLIA), for example the manual Orion RIA is able to recognise intact P1NP (trimeric form), whilst the automated Roche Elecsys 2010 analyser detects total P1NP (trimeric form plus thermal degradation forms of P1NP-

fragments excluded), therefore the former underestimates P1NP levels (Hlaing & Compston, 2014). Other advantages of the automated Roche Elecsys over manual RIA include high volume analysis and enhanced reproducibility (Garnero et al., 2008; Hlaing & Compston, 2014).

2.5.4.1.2 BALP

BALP is an abundant enzyme that is attached to the membrane of osteoblasts (Vasikaran et al., 2011). BALP plays an important role in the mineralisation of bone matrix (Harris, 1990) by catalysing the hydrolysis of inorganic pyrophosphate, which inhibits mineralisation. BALP allows the release of inorganic phosphate for hydroxyapatite formation (Wennberg et al., 2000). Alkaline phosphatase isoforms originate from a number of tissues predominately liver and bone, with bone isoforms accounting for approximately 50% of all isoforms in healthy individuals. The BALP can be separated from the other isoforms and detected by commercially available assays (Vasikaran et al., 2011); however, the cross-reactivity of the bone isoform with the liver isoform is high (up to 20 %) and limits its usefulness in patients with liver disease (Lombardi et al., 2012a).

2.5.4.1.3. OC

OC is the most ubiquitous, non-collagenous protein of the bone matrix. Its synthesis, by osteoblasts during bone formation, involves a precursor molecule with three glutamic acid (Glu) residues (Lombardi et al., 2015b). The γ-carboxylation of Glu residues by a carboxylase in osteoblasts alters the conformation of OC allowing its binding with hydroxyapatite (Lombardi et al., 2015b). OC can also bind to other proteins, receptors and cell surfaces, properties to facilitate its critical role in extracellular organisation. OC can also be decarboxylated or undercarboxylated in the circulation, where it is involved in a range of roles including energy metabolism (Lee et al., 2007), insulin sensitivity and secretion (Hwang et al., 2012), angiogenesis and reproduction (Oury et al., 2011). OC in the circulation contains intact OC, as well as large, medium and small size fragment (Hlaing & Compston, 2014). Smaller fragments are the breakdown products reflective of bone resorption (Lombardi et al., 2015b). Thus, OC may indicate overall alterations in bone turnover, rather than purely bone formation. OC is excreted by the kidneys and therefore, fragments can also be determined in the urine (Vasikaran et al., 2011). The heterogenous nature of OC fragments limit the clinical applications of OC. Available analytical methods for OC include automated or manual RIA, ELISA or ECLIA. Assays that recognise the intact OC and large fragments of OC show the greatest reproducibility and may be more

relevant to the measurement of bone formation (Lee et al., 2000; Hlaing & Compston, 2014).

2.5.4.2. Markers of bone resorption

2.5.4.2.1. N- and C-terminal cross-linked telopeptides of type I collagen (NTX and CTX)

NTX and CTX originate from the enzymatic cleavage of amino-terminal and carboxyl-terminal ends of type I collagen. There are different CTX isoforms; α if it is not isomerised or β if it is isomerised, with the β -isomerisation being more specific to mature collagen (Wheater et al., 2013). Both NTX and CTX can be measured in either the urine or serum and, notably, β-CTX can also by analysed in ethylenediaminetetraacetic acid (EDTA), which optimises its stability (Vasikaaran et al., 2011). β-CTX, determined in serum/plasma, is the preferred measure of bone resorption (Vasikaaran et al., 2011) (please see section 2.5.4.5) and can be analysed using ELISA, RIA and ECLIA methods (manual or automated) (Hlaing & Compston, 2014). NTX and CTX concentrations obtained in urine samples should be corrected for creatinine, and thus, pre-analytical and analytical variability of creatinine analysis may add another source of error in the results (Vasikaaran et al., 2011). For example, inter-individual and intraindividual variability of urinary NTX have been reported 15 and 26% (Banfi et al., 2010).

2.5.4.2.2. Pyridinoline (PYD) and Deoxypyridinoline (DPD)

Both crosslinks, PYD and DPD, act as molecular bridges that connect collagen molecules. During collagen degradation, these bridges undergo enzymatic processing to free collagen molecules. Differences in specificity of PYD and DPD have been observed, with DPD being more bone specific and almost exclusively derived from bone (Lombardi et al., 2012a). PYD and DPD are released in free and peptide-attached forms into the urine, where their concentration can be determined by a reverse phase high performance liquid chromatography or automated immunoassays detecting free and/or peptide-attached forms (Hlaing & Compston, 2014). The use of PYD and DPD as bone resorption markers is marred by challenges in controlling sampling conditions (*i.e.*, second void or 24h sampling) and the requirement to adjust urinary measures for creatinine (Vasikaran et al., 2011).

2.5.4.2.3. Tartate-resistant acid phosphatase 5b (TRACP5b)

Acid phosphatases are a big family of metalloenzymes, present in a number of tissues including bone, spleen, lungs, placenta prostate, erythrocytes, macrophage and platelets. The

acid phosphatase in bone, spleen and lungs display resistance to L (+) tartrate. The *b* form of isoform 5 (TRACP5b) is an osteoclast-specific isoform (Oddie et al., 2000). During bone resorption, osteoclasts release TRACP5b, which produces reactive oxygen species to digest bone breakdown products. Thus, TRACP5b was initially thought to indicate osteoclastic activity (Hannon et al., 2004). However, recent developments suggest that TRACP5b is informative about the number of osteoclasts, rather than their activity (Rissanen et al., 2008). The measurement of TRACP5b can be conducted using enzymatic and immunoassay techniques, however, they are often limited by the ability of the enzyme to bind to a2-macroglobulin, which decreases its binding with assay antibodies (Oddie et al., 2000).

2.5.4.3. Emerging BTM

Emerging BTMs have been used alongside existing, well-established BTMs. For recent reviews, please see Wheater et al. (2013), Garnero et al. (2014) and Chapurlat & Confavreux (2016). These emerging BTM can be classified into different categories including non-collageneous proteins of bone matrix (*e.g.,* osteopontin), regulators of osteoclast and osteoblast cell differentiation and activity *(e.g.,* OPG/RANKL, Wnt signalling pathway, sclerostin, *β*-catenin, periostin) and markers of bone matrix processes (*e.g.,* Type I collagen isomerisation, post-translational modification of non-collagenous proteins). For example, as mentioned in section 2.3.3., sclerostin is secreted by osteocytes to inhibit Wnt signalling pathway through its binding to LRP5/6 (Li et al., 2005). Thus, sclerostin blocks Wnt effects on osteoblasts and decrease bone formation. Sclerostin is responsive to mechanical loading, with decreases concentrations shown after involvement in weight bearing activities and increased concentrations shown following bed rest (Spatz et al, 2013; Belavy et al, 2016). Despite its relevance with bone formation, its high analytical and biological variability limits its current use in clinical settings and requires further study (Wheater et al., 2013).

2.5.4.4. Factors influencing BTMs

BTMs are influenced by a considerable number of controllable (*e.g.,* exercise, nutrition, circadian rhythm) and uncontrollable biological factors (*e.g.,* age, sex, ethnicity) (Table 2.2.). The effects of the controllable factors can be reduced by standardising sampling conditions; for example, collection of biological samples early in the morning, after an overnight fast or absence of systematic exercise 24 hours before collection. Uncontrollable factors should be taken into consideration when deciding about characteristics of the study population (Banfi et al., 2010; Lombardi et al., 2012a; Vasikaran et al., 2011). For example, postmenopausal women experience accelerated bone turnover and thus, they have higher BTM levels compared to their premenopausal counterparts (Khosla et al., 1997). BTMs have

been reported elevated up to one year after sustaining a fracture (Veitch et al., 2006), making it important to exclude participants with recent fractures in studies that investigate the effectiveness of dietary and exercise interventions.

2.5.4.4. Reference BTMs

Considering the characteristics of each BTM and its analytical and biological variability, it is apparent that each BTM has advantages and disadvantages. In 2012, the International Osteoporosis Foundation and International Federation of Clinical Chemistry published reference standards for BTM use in clinical research (Vasikaran et al., 2011). The selection criteria for BTM reference standards were: adequate characterisation and clear definition of the BTM; bone-specific origin; prediction of fracture risk; monitoring of osteoporosis therapy; widespread availability of the methodology (if possible automated); satisfactory levels of biological and analytical variability; ease of sample handling and analysis; good stability and ability to be measured in blood (Vasikaran et al., 2011). These recommendations were based on meta-analyses of BTM studies that were used to predict fracture risk, cohort studies following pre-analytical warnings and clinical trials. CTX (serum or plasma) and P1NP (serum or plasma) were chosen as the reference standards for bone resorption and formation (Vasikaran et al., 2011).

CTX is considered to satisfactorily reflect bone resorption (*e.g.,* compared to TRACP5b that may reflect osteoclast number), although it is non-specific for bone as are all the available markers of bone resorption. CTX is measureable in both serum and EDTA plasma (*e.g.*, compared to PYD and DPD that are determined mainly in urine) and automated or manual assays are widely available. Its biological and analytical variability and sampling stability are well characterised allowing standardisation of sampling and handing conditions. Importantly, unlike other bone resorption markers, CTX responds to anti-resoptive treatment (serum NTX is less responsive than CTX to therapy) (Vasikaran et al., 2011).

P1NP reflects bone formation to an acceptable level (compared to OC that may reflect bone turnover), has predominantly bone origin (none of the currently available markers is specific to bone only), is affected by hormones to a lesser extent than others (P1CP clearance is sensitive to thyroid hormones and IGF-1). From an analytical perspective, P1NP can be measured in either serum or plasma (EDTA), and currently available automated or manual assays show good precision (*i.e.*, BALP- up to 20% cross-reactivity with liver isoforms) and low analytical CV (<10%) (Vasikaran et al., 2011). P1NP is superior in monitoring

osteoporosis treatment, for example, P1NP concentrations decrease up to 80% from baseline following anti-resoptive treatment (Brown et al., 2009).

Table 2.2 Controllable and uncontrollable factors influencing BTMs (modified from Vasikaran et al., 2011).

Source	Significance	Direction of Effect	Indicative reference
Non-Controllable factors			
Age	Very important	Infants, children, adolescents and older individuals have higher levels of BTMs compared to adults.	Khosla et al., 1997
Ethnicity	Not important	Small differences *i.e.,* lower BTMs levels in African American compared to Caucasians	Finkelstein et al., 1996 Henry et al., 2000
Sex	Very Important	Age dependent sex-differences. Higher BTMs in older women than men	Khosla et al., 1998
Immobility/ Bedrest	Important	Markers of bone resorption increase. Markers of bone formation decrease or remain unaltered.	Zerwekh et al., 1998
Genetics	Very important	Strong correlations in BTMs between twins	Donescu et al., 2007
Drugs	Important	Corticosteroids, aromatase inhibitors, antiepileptic drugs, thiazolidinediones, statins, heparin Well-established effects for corticosteroids (decreased BTM) and anticonvulsants (increased BTM)	Szulc & Delmas, 2008 Vasikaran et al., 2011
OCP	Somewhat important	The effects of OCP on BTMs are of particular interest for the current programme. Lower BTM in OCP users compared to controls.	Hermann & Seibel, 2010
Diseases	Important	Often increased BTMs with disease including liver disease, renal impairment, thyroid disorders, systemic inflammatory disease, diabetes, degenerative joint disease-	Seibel et al., 2005
Fracture	Important	Elevated BTMs up to 1 year after the injury	Veitch et al., 2006
Pregnancy and lactation	Important	Elevated BTM levels during pregnancy (third semester) and breastfeeding.	Black et al., 2000 Ulrich et al., 2003
Controllable factors			
Menstrual cycle	Somewhat important	Small decreases in bone resorption and increases in bone formation during luteal phase.	Zittermann et al., 2000 Gorai et al., 1998
Circadian rhythm	Very important	Bone resorption markers display great variability -peak early in the morning and nadir in the afternoon. Bone formation markers are affected less than bone resorption markers.	Qvist et al., 2002 Sclemmer et al., 1992
Fasting/ nutrient intake	Very important	Feeding (*i.e.,* glucose, calcium, protein, fat or a mixed meal) decreases bone resorption Bone formation markers are less affected by feeding than markers of bone resorption. Great variability among BTMs.	Henriksen et al., 2003 Schlemmer et al., 1997 Clowes et al., 2002
Exercise	Very important	Alterations depending on the type, intensity and duration of exercise.	Woitge et al., 1998 Welsh et al., 1997

OCP: oral contraceptives pill; BTM: bone turnover marker

2.6. Key determinants of bone health

Genetics (Ralston & Uitterlinden, 2010; Ferrari, 2008), sex steroids (Manolagas et al., 2013; Clarke & Khosla. 2010; Khosla et al., 2012), nutrition (Rizzoli, 2008; 2014) and mechanical loading (Robling et al., 2006; Bonet & Ferrari, 2010) are key influencers of bone health. We acknowledge that genetics have well established influences on bone health; a number of bone phenotypes including BMD, bone architecture, bone turnover, bone loss and fracture risk have a genetic background (for reviews please see Ralston & Uitterlinden, 2010; Ferrari, 2008; Uitterlinden et al., 2013). Non-skeletal characteristics that influence bone phenotypes such as BMI, muscle strength, age at menopause, falls and protective responses may also have their own genetic basis (for reviews please see Ralston & Uitterlinden, 2010; Ferrari, 2008; Uitterlinden et al., 2013). This literature review focuses on sex steroids, nutrition and mechanical loading due to their relevance with the intervention and the study populations of this thesis.

2.6.1. Sex steroids and bone

Both male and female sex steroids, namely androgens and oestrogens, are critical for bone acquisition during puberty and for bone mass maintenance in adulthood (Vanderschueren et al., 2014; Khosla et al., 2012; Manolagas et al., 2013). Sex steroids act on bone by binding to the receptors located on bone cells; oestrogens bind to estrogen receptor (ER) α or ER-β and androgens bind to the androgen receptor (AR) (Beato & Klug, 2000). Importantly, the greatest proportion of oestrogens and testosterone are tightly bound to sex hormone binding globulin (SHBG) (40-65%), which limits the bioavailability of these steroids (Khosla & Pacifici, 2013). In contrast, oestrogen and testosterone in the free or bound to albumin form are easily accessible (Khosla & Pacifici, 2013). Androgens can be converted into oestrogens by the P450 aromatase enzyme complex, providing the mechanism to support that oestrogen may mediate at least some of the effects of androgens in men (Vanderschueren et al., 2014; Manolagas et al., 2013). Analogously, androgens are also produced by females (ovarian and adrenal secretion and peripheral conversion of weak androgens to testosterone) and may influence the female skeleton, although these actions are less well established (Khosla & Pacifici, 2013).

2.6.1.1. Normal menstrual function

Menstrual function is controlled by the hypothalamic-pituitary-ovarian axis (Figure 2.4). Gonadotropin-releasing hormone (GnRH) secretion occurs in a pulsatile way by the hypothalamus and triggers the release of luteinising hormone (LH) and follicle stimulating hormone (FSH) by the anterior pituitary gland. LH and FSH stimulate follicular

development and the production of oestrogen from the ovaries, which provides positive feedback to the anterior pituitary. Once oestrogen concentrations reach a threshold, a surge in LH levels allows ovulation to take place (Figure 2.5.). Following ovulation, the formation of the corpus luteum increases progesterone levels to support the hyperplasia of the endometrium in the event of implantation. In the absence of pregnancy, the corpus luteum degenerates and progesterone and oestrogen levels decrease. The endometrial lining is shed and menstruation is initiated (Clarke & Khosla, 2010). The menstrual cycle is defined as the period from the onset of menstruation to the day prior to the onset of the next menstruation, has a typical duration of 28 days ±7 days and is usually categorised into 3 phases, follicular, ovulatory and luteal (Figure 2.5.). The occurence of consistent monthly cycles is termed eumenorrhea.

The increase in oestrogen secretion with menarche (first menstrual cycle), typically between the ages of 11–13 years, promotes rapid longitudinal and radial skeletal growth for the next decade or so. Importantly, puberty is characterised by accelerated BMD gains, whilst slower increases in BMD and consolidation occur during late adolescence and young adulthood, until peak bone acquisition is achieved (Clarke & Khosla, 2010). The normal menstrual cycle, with its cyclic changes in reproductive hormones, plays a critical role in bone maintenance in adulthood until the menopause, when the decline in oestrogen results in rapid bone loss (Khosla and Pacifici, 2013; Manolagas et al., 2013).

Oestrogens have direct and indirect effects on bone resorption through the expression of RANKL (expressed by osteoblasts, T- and B-lymphocytes), OPG (expressed by osteoblasts) and the production of IL-1, IL-6, TNF-a, M-CSF and prostaglandins (acting on osteoblasts and bone marrow stromal cells) to modulate osteoclast differentiation, activity and survival (Khosla et al., 2012; Clarke & Khosla, 2010). Oestrogen-dependent regulation of bone formation is mediated by alterations in osteoblast activity apoptosis and alterations in oxidative stress (Khosla et al., 2012). Some oestrogens actions on the skeleton may include control of intestinal calcium absorption and renal calcium reabsorption (Gennari et al., 1990; McKane et al., 1995), although these mechanisms are still debated (Manolagas et al., 2002).

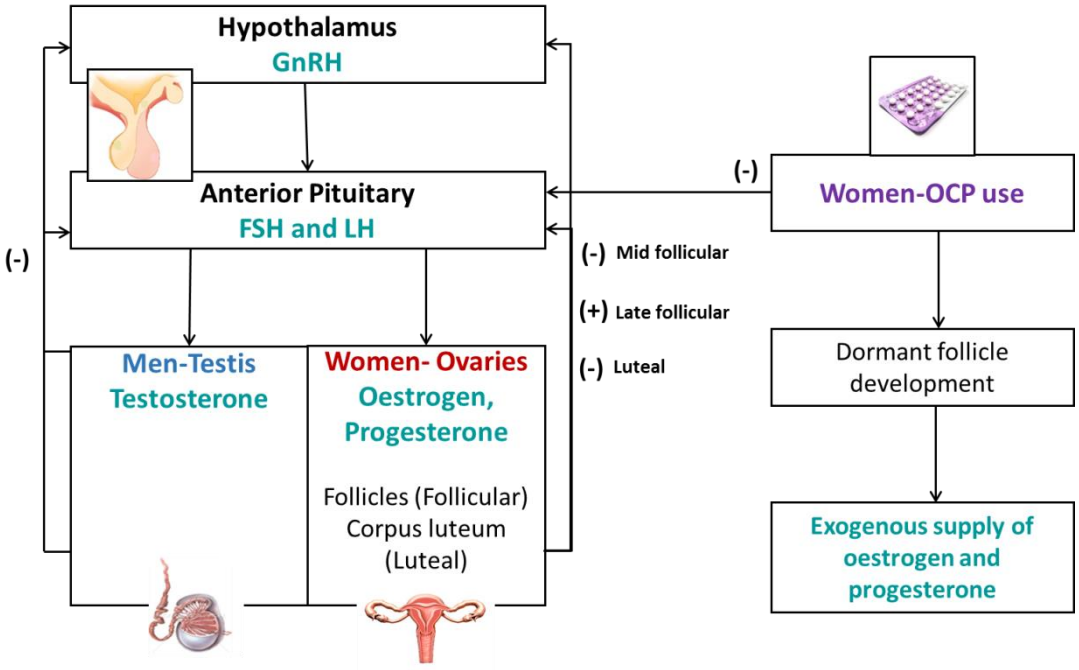


Figure 2.4. The control of endogenous sex hormones in eumenorrheic women (dark red), in men (blue) and OCP users (purple). GnRH secretion occurs in a pulsatile way by the hypothalamus and triggers the release of LH and FSH by the anterior pituitary gland in both sexes. In women, LH and FSH stimulate follicular development and the production of oestrogen from the ovaries. Generally, oestrogen and progesterone exert negative feedback on the anterior pituitary, resulting in reduced release of FSH and LH. During the late follicular phase, the rapid elevation in oestrogen concentrations exerts positive feedback on the anterior pituitary resulting in the LH surge (for more details about the changes of the concentrations of these hormones throughout the menstrual cycle, please also see Figure 2.5.). LH surge allows the release of the oocyte from the dominant follicle and initiates ovulation. Under the action of LH, the dominant follicle is luteinised to form the corpus luteum, which produces progesterone and oestrogens. In combined OCP users, the exogenous provision of oestrogen and progesterone, provide negative feedback on the hypothalamus and pituitary gland, supressing FSH, LH and GnRH. In men, LH and FSH pulsatile release by the anterior pituitary determines testicular testosterone secretion, which in turn exerts negative feedback on the anterior pituitary and hypothalamus, resulting in reduced release of LH, FSH and GnRH. FSH: follicle-stimulating hormone; LH: luteinizing hormone; GnRH: Gonadotropin-releasing hormone; OCP: oral contraceptive pill; (+): positive feedback; (-): negative feedback Adapted from Burrows & Peters, 2007; Clarke & Khosla, 2010; Jiménez-Reina et al., 2016.

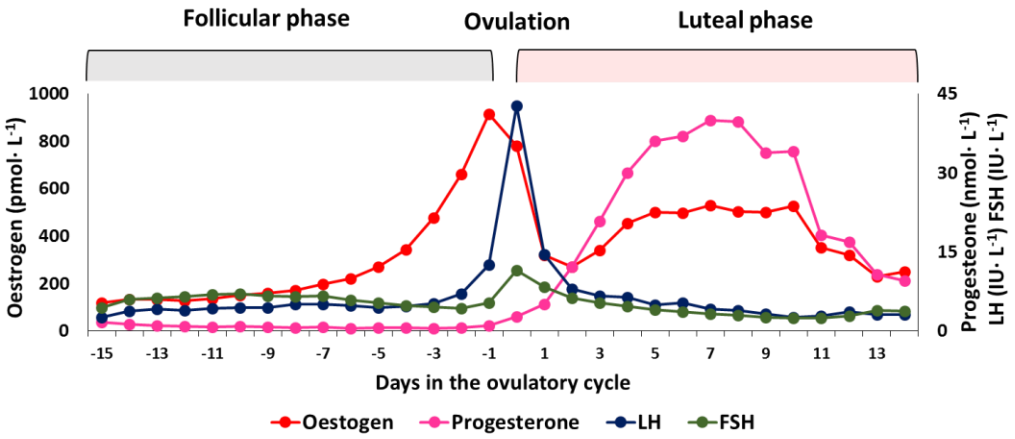


Figure 2.5. Profile of the main reproductive female hormones during a normal menstrual cycle. Classic characteristics include the oestrogens peak in the late follicular phase followed by the LH surge and the elevation of progesterone levels during the luteal phase. FSH: follicle-stimulating hormone; LH: luteinizing hormone. Graph constructed using the data from Stricker et al., 2006.

2.6.1.2. Premenopausal menstrual disturbances

Studies have demonstrated that age of menarche is an important determinant of bone growth and BMD (Sowers et al., 1992; Chevalley et al., 2005). Later age at menarche is a risk factor for low BMD, premenopausal bone loss and bone injury (Waugh et al., 2009; Bennell et al., 1999b). Exercise-associated menstrual disturbances commonly occur among physically active women and vary from subclinical menstrual disturbances, including luteal phase defects and anovulation, to severe clinical manifestations of oligomenorrhea and amenorrhea. Luteal phase defects and anovulation often go undiagnosed, as they are often not accompanied by changes in menstrual cycle length, yet research suggests that they are the most frequent type of exercise-associated menstrual disturbances (De Souza et al., 2010). In contrast, oligomenorrhea (long, irregular menstrual cycles of variable duration between 36 to 90 days) and amenorrhea (absence of menses for at least 3 months) are severe, clinical representations of hypogonadism (De Souza et al., 2010). The basic characteristics and hormonal characteristics of these disorders are presented in Figure 2.6. Research shows that amenorrheic women have very low concentrations of oestrogens that are accompanied by increases in markers of bone resorption, low BMD scores and negative alterations in bone geometry and microarchitecture (De Souza et al., 2008; Cobb et al., 2003; Christo et al., 2008; Ackerman et al., 2011; 2012a). These findings are similar to those from studies in amenorrheic women with anorexia nervosa that show that osteoporosis is a well-established consequence of this condition (Misra & Klibanski, 2011; 2014). Bone health is also

compromised in oligomenohheic atheletes compared to their eumenorrheic counterparts (Cano Sokollof et al., 2015). The effects of the subclinical exercise-associated menstrual disturbances are less clear; with no apparent reductions in BMD (De Souza et al., 2003; 1997), which is in line with the milder perturbations in reproductive hormones. Future research is needed to explore if other parameters of bone health such as bone microarchitecture, BTM and strength are affected.

Another condition that suggests an important role of sex hormones in premenopausal women is polycystic ovary syndrome, which is accompanied by follicular arrest, poly-ovarian cysts and an LH-dependent elevation in androgen release (Kassanos et al., 2014; Zborowski et al., 2001). Women with polycystic ovary syndrome have higher BMD and augmented cortical bone properties compared to age-matched controls (Kassanos et al., 2014; Zborowski et al., 2001). This model provides some evidence of the effects of androgens on the female skeleton, although potential confounding factors such as high body mass index (BMI), body composition (*i.e.,* high fat mass) and menstrual irregularities (oligo- and amenorrhea) may also influence bone in an independent way in these studies.

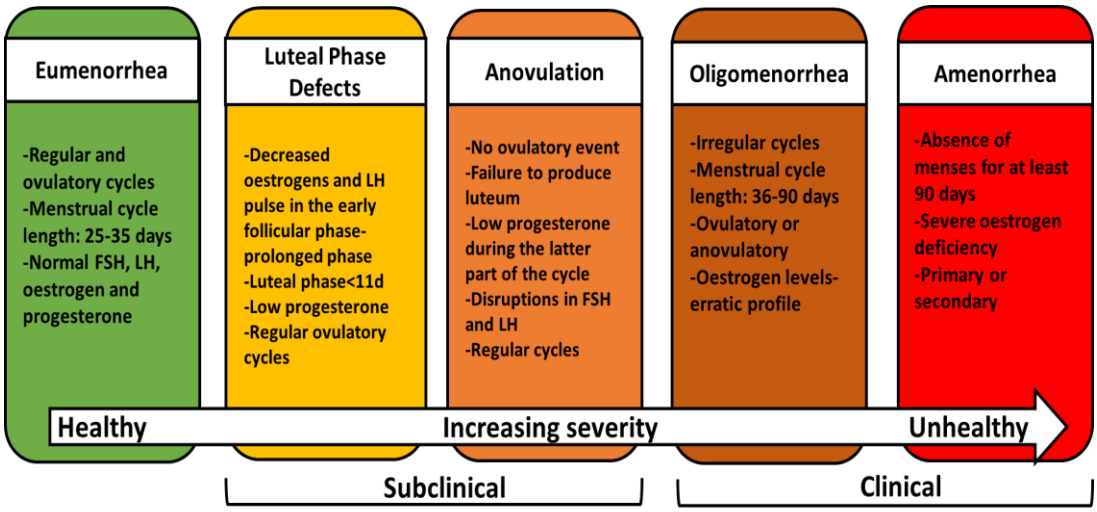


Figure 2.6. Spectrum of menstrual function from eumenorrhea to amenorrhea. Primary amenorrhea: failure to menstruate by 15 years in girls with secondary sex characteristics; Secondary amenorrhea: abnormal cessation of menses after menarche. Adapted from De Souza, 2003.

2.6.1.3. The menopause

The reduction in oestrogen levels accompanying the menopause is associated with an accelerated bone loss, low bone strength and an increased fracture risk (Riggs et al., 1998). Postmenopausal bone loss results from increased bone turnover rate as suggested by

increases in both bone resorption and formation (Manolagas et al., 2013). However, the increase in bone resorption outweighs that of bone formation, resulting in uncoupling of bone turnover and net bone loss (Manolagas et al., 2013). Bone loss occurs in both the trabecular and cortical bone; however, cortical thinning initiated with menopause is greater than trabecular thinning and results from an increase in the medullary diameter and endocortical resoprtion (Ahlborg et al., 2003; Bartell et al., 2013). Conversely, periosteal apposition may, to some extent, preserve bone strength (Manolagas et al., 2013). The effects of oestrogen deficiency on bone are mediated directly by the genesis, activity and apoptosis of osteoblasts and osteoclasts (please see section 2.6.2.1), but also through a downregulation of oestrogen receptor alpha (ER-α) expression, which attenuates the responsiveness of osteoblasts to mechanical loading. Within a decade after menopause, the rate of bone loss slows and matches that seen in older men (Clarke & Khosla, 2010). The slower bone loss at this phase is explained by ageing-related mechanisms and characterised primarily by loss of cortical bone and increased intracortical porosity (Manolagas et al., 2013).

2.6.1.4. OCP

The term hormonal contraceptives includes injected contraceptives, OCP and vaginal rings (Burrows & Peters, 2007). The progestin-only contraceptive injection depot medroxyprogesterone acetate known as DMPA is a highly effective contraceptive that does not require compliance on a daily or weekly basis. DMPA injection provides low doses of progesterone, which becomes available to the systemic circulation. The use of DMPA have been demonstrated to have negative effects on bone health (Curtis & Martins, 2006). Combined OCP, the most common type of OCP (Lader, 2009); contain a synthetic oestrogen (*i.e.*, ethinyl oestradiol) and a progesterone derivative. The biological characteristics of the latter component depend on its potency and its relative binding affinity (Carr, 1998). Combined OCP can be prescribed as monophasic, biphasic, or triphasic formulations; with differences depending on whether hormones are delivered in constant (monophasic) or varying amounts (diphasic and triphasic) across the active pill phase (usually 21 days). Modern combined OCP formulations contain low doses (20–35 $\mu g \cdot d^{-1}$) and ultra-low doses (15 $\mu g \cdot d^{-1}$) of oestrogen compared to earlier higher dose preparations ($\geq$50 $\mu g \cdot d^{-1}$) (Burkmann et al., 2011). Combined OCP, through exogenous oestrogen and progesterone supplementation, provide negative feedback on the hypothalamus and pituitary gland, supressing FSH, LH and GnRH (Burrows & Peters, 2007) (Figure 2.4.). Low levels of LH and FSH inhibit the genesis of follicles and ovulation and down-regulate the endogenous production of oestrogen and progesterone. Thereby, cyclical changes in the uterus and ovaries are obstructed and pregnancy is prevented.

Despite the great number of studies in the area of combined OCP and bone health, the results are mixed at least in part due to the wide range in the available formulations and brands and dependent on age and menstrual status. In adult, non-exercising women, OCPs have little effect on BMD (Liu & Lebrun, 2006). In peri- and postmenopausal women, there is evidence to support a positive effect of OCPs (and by inference low circulating sex hormone levels) on BMD (Kleerkopper et al., 1991; Liu & Lebrun, 2006). In contrast, in adolescents and young adults still gaining bone, OCPs may slow down bone development and growth. In this age group, suppressed BTM response and reductions in BMD acquisition compared to BMD gains in age-matched controls have been reported (Cibula et al., 2012; Polatti et al., 1995; Pikkarainen et al., 2008). These effects appear to be greater among users of ultra-low dose formulations (Cibula et al., 2012). OCP use may also have unfavourable effects on bone health compared to exercising women with regular, ovulatory menstrual cycles. Physically active women taking low-dose OCPs had lower BMD compared to physically active non-users (Hartard et al., 1997; Weaver et al., 2001) or sedentary women (Weaver et al., 2001). These effects may be at least partially related to the potential reduction in oestrogen levels achieved by OCP administration. Furthermore, OCP use is often accompanied by further hormonal changes including decreases in IGF-1 and increases in IGF-1 binding proteins and T_3 levels (Blackmore et al., 2011; Wiegratz et al., 2003; Hansen et al., 2009), which exert actions on bone (Giustina et al., 2008; Huang et al., 2000; Lakatos et al., 2000). Lastly, OCP are prescribed for the regulation of menstrual function in exercise-related menstrual disturbances or amenorrhea in women with anorexia nervosa; although their use is not recommended as first line of treatment (De Souza et al., 2014a). Current evidence suggests OCP alone may be ineffective at correcting BMD in amenorrheic athletes or amenorrheic patients with anorexia nervosa, particularly if not combined with non-pharmacological options (*i.e.,* increased dietary intake and/or reduced exercise, weight gain) (Fredericson & Kent, 2005; De Souza et al., 2014a; Fazeli and Klibanski, 2014). As such, major guidelines do not suggest the use of OCP for BMD improvements (Female Athlete Triad Coalition and ACSM-De Souza et al., 2014a; Nordic Federation of Societies of Obstetrics and Gynaecology-Bergstrom et al., 2013).

2.6.1.5. Sex steroids in men

In men, the main androgen is testosterone, which is controlled by the hypothalamic-pituitary- testicular axis. Similar to women, GnRH stimulates FSH and LH pulsatile release by the anterior pituitary, which in turn, determines testicular testosterone secretion (approximately 95% of testosterone production) (Feldman et al., 2002; Jimenez-Reina et al., 2016) (Figure 2.4.). Androgens act directly on chondrocytes to increase ossification of

growth plates and thus, linear bone growth. Actions of androgens related to bone formation include increased calcium absorption and retention, stimulation of osteoblast progenitors' proliferation, reduced osteoblast apoptosis and increased periosteal bone formation (Lorentzon et al., 2005; Vanderschueren et al., 2014; Carnevale et al., 2010). Some of their effects on osteoclasts include RANK expression in pre-osteoclasts and regulation of osteoclast activity and survival (Manolagas e al., 2013). Indirect effects, through their impact on muscle (Vandershuerren et al., 2003) and regulatory role on hormones [*e.g.*, growth hormone (GH)] cytokines and growth factors at a local level (*e.g.*, TGF-β, IGF-1, and IL-6) (Manolagas et al., 2013) have also been demonstrated.

Oestrogens are also critical for bone health in men and may mediate at least some of the effects of androgens on bone (Manolagas et al., 2013; Vanderscueren et al., 2014). Indeed, both oestrogen and testosterone are needed for bone growth in men (Vanderscueren et al., 2014; Khosla & Pacifici, 2013). At low circulating levels of oestrogen, such as those experienced by pubertal males, oestrogen acts synergistically with testosterone to enhance periosteal apposition, whereas at higher levels of oestrogen, such as those experienced by females during later puberty, periosteal apposition is inhibited. The understanding of the role of oestrogen in the male skeleton has progressed by observations of male patients with impaired responsiveness of bone to oestrogen (*e.g.*, mutations in ER-α gene) or impaired oestrogen synthesis (*i.e.*, aromatase deficiency). Both models are characterised by low BMD and unfused epiphyses despite normal testosterone levels (Smith et al., 1994; Carani et al., 1997). Oestrogen replacement in these patients increases bone mass, further supporting the role of oestrogen on the male skeleton (Bilezikian et al., 1998). In the general population, Khosla et al. (1998), conducted a cross-sectional study of 314 men aged 23-30 years and found that bioavailable oestradiol, and to a lesser extent testosterone, were the most consistent predictors of BMD at several skeletal sites. Subsequent experiments evaluated the relative importance of oestrogens and androgens on bone turnover in younger (Leder et al., 2003) and elderly men (Falahati-Nini et al., 2000). Both androgens and oestrogens were reported to mediate bone resorption independently in younger men (Leder et al., 2003). In elderly men, oestrogens were the main regulators of bone resorption, whilst both oestrogens and testosterone contributed to the maintenance of bone formation (Falahati-Nini et al., 2000). Taken together, current evidence suggests an essential role of oestrogens and androgens in bone homeostasis in the male skeleton.

2.6.2. Nutrition and bone

Several aspects of nutritional intake, including sufficient caloric intake (energy), macronutrient intake (protein, carbohydrates, fat) and micronutrient inakte (*e.g.*, calcium and Vitamin D), are essential for bone metabolism and health (Ilich & Kersetter, 2000; Rizzoli et al., 2008). These nutritional factors also indirectly influence bone through the release of products resulting from their metabolism (*e.g.*, amino acids), modulation of circulating hormones (*e.g.*, calciotropic hormones, reproductive hormones) and changes in bone-related factors other than hormones (*e.g.*, body weight and body composition) (Rizzoli, 2008; Heaney, 2013). This section will review the effects of prominent nutritional factors on bone health.

2.6.2.1. Energy

Both inadequate and excessive dietary energy intake have been associated with alterations in bone measures (Shapses & Sukumar, 2012; Fazeli & Klibanski, 2014). Anorexia nervosa, an extreme example of inadequate dietary energy intake, is accompanied by a reduction in BMD, altered bone microarchitecture (i.e., decreased cortical thickness and decreased trabecular volume, number and thickness) and increased fragility risk (Fazeli & Klibanski, 2014; Misra & Klibanski, 2011). Less severe caloric restrictions have also been associated with bone loss in non-obese populations (Caporaso et al., 2011; Villareal et al., 2006; 2016) and physically active individuals (Nattiv et al., 2007; Ihle & Loucks, 2004). For example, a cross-sectional study of 52 non-obese women, classified by daily caloric intake at 100%, 80%, or 55% of their recommended daily energy requirement, showed a significantly lower femoral BMD (-10%) in the group with the lowest caloric intake (55%) and a trend toward lower spine areal BMD compared to the other two groups (Caporaso et al., 2011). Reductions in caloric intake and disordered eating have also been reported to compromise bone health (Cobb et al., 2003; Barrack et al., 2008; Nieves, 2010; 2016) of physically active women in line with the Female Athlete Triad (Nattiv et al., 2007; De Souza et al., 2014a) (for a detailed description please see 2.9 and 2.11.2). The effect of caloric restriction on bone is poorly understood and the relative contribution of diet-induced energy deficiency versus other nutritional deficiencies is currently unknown.

Some studies suggest that obesity is associated with increased bone mass and that a greater body weight is protective against bone loss (Felson et al., 1993; Maimoun et al., 2016). In a cross-sectional study, Evans et al. (2015) examined site-specific BMD, bone structure and bone strength in obese and normal-weight young (25-40 y) and older (50-75 y) individuals. They showed lower BTM and higher BMD at all skeletal sites, favourable bone

microarchitecture (greater cortical thickness and volumetric BMD and greater trabecular number) and greater bone strength at the distal radius and distal tibia in the obese participants, with these differences being more apparent in the older group (Evans et al., 2015). However, despite such evidence of a positive impact of obesity on bone, there is increasing evidence that excess weight due to adiposity may also result in low BMD (Greco, et al. 2010), poor bone quality and increased fracture risk (Compston, et al., 2011; Nielson et al., 2012) across a range of age groups. These negative effects appear to be more profound at high BMI values (Shapses & Sukumar, 2012; Nielson et al. 2012).

Bone loss often accompanies weight loss in overweight and obese individuals (Shapses & Sukumar, 2012). A recent systematic review and meta-analysis conducted by Zibellini et al. (2015) included 41 studies with diet-induced weight loss intervention of 2-24 months in overweight and obese populations. They showed a small significant decrease in total hip BMD but no change in lumbar spine BMD together with modest changes in BTM early in the interventions (2-3 months), indicative of bone breakdown (Zibellini et al., 2015). The weight loss achieved in randomised controlled trials is approximately 10% and is accompanied by BMD reductions of about 2% (Schafer, 2016).

Bariatric surgical procedures are classed as restrictive, restrictive/malabsorptive, and primarily malabsorptive and result in a 30% reduction in body weight (Schafer, 2016). Severe reductions in BMD (approximately 10%) and strength, together with changes in bone microarchitecture (*i.e.,* reduced trabecular number) have been reported, with the magnitude of these alterations varying depending upon the surgical procedure (Hsin, et al. 2015; Frederiksen, et al. 2016). Although bone loss has been observed post-surgery, the long-term effects of these changes and the incidence of osteoporosis and fracture risk in these patients are still unknown (Scibora et al., 2012; Scibora, 2014).

2.6.2.2. Protein

Dietary proteins serve as sources of essential amino acids for the maintenance of bone structure. Protein intake also affects bone through alterations in bone trophic factors such as IGF-1, calcium absorption and other physiological changes (*e.g.,* maintenance or reduction of muscle mass) (Rizzoli et al., 2010; Bonjour, 2005). Dietary protein contains sulfur amino acids and their oxidation contributes to acid production and a decrease in pH, which may influence the balance between bone formation and resorption and induce urinary calcium excretion (Bonjour et al., 2005; Kerstetter et al., 2011). However, there is little evidence to support a negative association between protein and bone health (Bonjour et al., 2005). The

vast majority of epidemiological, cross-sectional and randomised controlled studies have demonstrated a positive effect of a protein intake on BMD (Conigrave et al., 2008; Darling et al., 2009; Rizzoli et al., 2008). A systematic review and meta-analysis conducted by Darling et al. (2009) on protein supplementation and bone health demonstrated a small positive impact of protein supplementation on lumbar spine BMD but no significant effect on fracture rate. A high-protein diet is a common dietary prescription for weight loss and weight maintenance (Leidy et al., 2007) and has been shown to attenuate BMD reductions accompanying weight loss in overweight and obese individuals (Skov et al., 2002; Thorpe et al., 2008).

2.6.2.3. Calcium

Calcium is an important component of bone mineralisation, with bone containing approximately 99% of total calcium in the body. Due to the great dependence of various cells and organs upon small variations in extracellular calcium levels, calcium levels are tightly controlled in the circulation by three main hormones: PTH, calcitonin and Vitamin D. PTH is the major regulator of calcium homeostasis. Elevated extracellular ionised calcium levels result in decreased PTH secretion from the chief cells of the parathyroid gland, whilst low levels of extracellular ionised calcium trigger an increase in PTH release. In the latter case, mobilisation of intracellular calcium is achieved through PTH/PTH-related peptide receptor-dependent mechanisms that activate G protein signals (Poole & Reeve, 2005). Calcitonin is secreted from the thyroid gland and antagonises the actions of PTH in calcium homeostasis (Mundy et al., 1999). Calcium is absorbed in the intestine and reabsorbed in the kidneys under the actions of Vitamin D (Mundy et al., 1999). There is a strong body of evidence linking calcium intake to skeletal growth (adequate calcium intake) and bone loss (inadequate calcium intake) (Bonjour et al., 1997; Matkovic & Heaney, 1992). Several studies have used calcium either in the form of supplements or through increased dairy consumption in children and adolescents and have shown positive influences in BMC and BMD compared to controls (Winzenberg et al., 2006; Rizzoli et al., 2008). Studies in young adults have shown a positive association between calcium intake and BMD (Nakamura et al., 2001; van den Hooven et al., 2015). Conversely, low calcium intake may be a risk factor for stress fracture injury (Nieves et al., 2010) and calcium supplementation (in combination with Vitamin D) in individuals with low calcium intake at baseline may reduce stress fracture risk (Lappe et al., 2008). In older adults, calcium supplementation may attenuate some of the age-related bone loss, with these effects being more profound in individuals with low calcium intakes of 500–1000 mg $\cdot d^{-1}$ at baseline. A meta-analysis of 23 trials and 41,419 participants showed that calcium supplementation alone or in combination with

vitamin D supplementation was associated with a 0.5% reduction in hip bone loss and a 1.2% reduction in spine bone loss (Tang et al., 2008). The same meta-analysis showed a 13% reduction in fracture risk with superior treatment effects when calcium and Vitamin D doses were high (calcium ≥ 1200 mg and Vitamin D ≥ 800 IU) (Tang et al., 2008).

2.6.2.4 Vitamin D

Vitamin D is an essential nutrient with several skeletal and non-skeletal roles (*i.e.,* anti-carcinogenic, immunological, neurological, cardio-protective, anti-diabetic) (DeLuca et al., 2004; Wacker & Hollick, 2013). Vitamin D can be either synthesised in the skin or consumed via diet and supplements. Vitamin D, from both sources, is inactive and needs to be hydrolysed twice to its biologically active form 1,25-$(OH)_2$D (Holick et al., 2011). The first hydroxylation occurs in the liver and converts Vitamin D to 25-hydroxyvitamin D (25-(OH)D) and the second hydroxylation takes place in the kidney converting 25-(OH)D to the biological active 1,25-$(OH)_2$D. Both 25-(OH)D and 1,25-$(OH)_2$D serve as markers of Vitamin D status. 1,25-$(OH)_2$D stimulates intestinal calcium and phosphorus absorption and calcium reabsorption from the kidneys. In bone, 1,25-$(OH)_2$D binds to its receptor present on the osteoblasts to regulate the expression of RANKL; which can then interact with its receptor RANK, or decoy receptor OPG, to promote or inhibit osteoclastogenesis and thus control bone resorption and calcium mobilisation from the skeleton (Holick et al., 2011). Poor vitamin D status reduces the efficiency of intestinal calcium and phosphorus absorption and results in an elevation in PTH levels in an attempt to maintain serum calcium levels (Mundy et al., 1999; Wacker & Hollick, 2013). Poor vitamin D status has been associated with low BMD in children (known as rickets) and adults (termed osteomalacia) (Gordon et al., 2008). In addition to these negative skeletal effects, Vitamin D deficiency contributes to muscle weakness (Gordon et al., 2008; Bischoff-Ferrari et al., 2009). In physically active individuals, suboptimal vitamin D status has been reported as a risk factor for altered bone turnover (Lutz et al., 2012; Evans et al., 2008) and stress fracture injury (please see 2.8.2) in some (Ruohola et al., 2006; McClung & Karl, 2010) but not all previous studies (Lewis et al., 2013). The impact of an increase in Vitamin D intake via supplementation or dietary sources on bone parameters remains unclear mainly due to differences in Vitamin D supplementation forms, co-supplemetantion with other micronutrients (e.g., calcium), study duration and particiapants' baseline Vitamin D status . Eight weeks of supplementation with 800 IU of vitamin D [as 1,25-$(OH)_2$D] in combination with 2000 mg of calcium reduced stress fracture incidence in female Navy recruits (Lappe et al., 2008), whereas supplementation with Vitamin D [as 25-(OH)D] for the same study duration (8-week) had no effect on BMD in elite female basketball players with low Vitamin D levels at baseline

(Bellows et al., 2013). Notably, potential side effects resulting from high doses of calcium (such as constipation, kidney stones, renal failure, and vascular calcification) and Vitamin D (anorexia, weight loss, and polyuria, increased calcium levels and cardiac arrhythmias) should be considered (Wesner, 2012).

2.6.3. Mechanical loading and bone

Compelling evidence supports the positive effects of mechanical loading in establishing bone mass during growth, conserving it during adulthood and mitigating bone loss in later life (Nikander et al., 2010; Weaver et al., 2016; Robling et al., 2006). Wolf (1892) first proposed that bone is able to adapt its mass and architecture to prevailing mechanical strain. Based on this theory, the mechanostast theory, developed by Frost (1990), describes the site-specific addition of bone in response to large amounts of strain and the removal of bone due to insufficient strain (Frost, 1987). Indeed, sports participation in weight-bearing activities results in increased bone mass and enhanced architecture compared to non-weight bearing activities or a sedentary lifestyle (Taafe et al., 1997; Olmedillas et al., 2012; Scofield & Hecht, 2012). Conversely, bone loss has been reported in humans and animals subjected to spaceflight or ground-based models for spaceflight (*e.g.*, bed rest) (Sibonga, et al. 2015; Spector, et al. 2009).

Mechanoreceptors are present on osteocytes and have the ability to detect strain-induced interstitial fluid flow, fluid shear stress and activate signalling pathways (*e.g.*, canonical Wnt pathway) and molecules (*e.g.*, sclerostin, IGFs, prostaglandin and nitric oxide) that control bone remodelling (Lombardi et al., 2015a; Bonnet & Ferrari, 2010). The indirect effects of mechanical loading on bone are mediated in part by muscle contractions and increases in lean body mass, which impose stresses to the bone that are detected by the mechanoreceptors (Lombardi et al., 2015a; Bonnet & Ferrari, 2010). Changes in circulating hormones and growth factors may also mediate the effects of mechanical loading, by altering bone turnover or mineral homeostasis (Lombardi et al., 2015a; Bonnet & Ferrari, 2010).

Osteogenic responses are initiated when bone is imposed to strains of greater magnitude than a threshold level, which is determined by a habitual strain range (Iwaniec & Turner, 2016; Weaver et al., 2016: Robling et al., 2006). Exercise based interventions have demonstrated bone gains, favourable alterations in bone architecture and increases in bone strength across a range of populations including children/adolescents (Meyer et al., 2011; Lofgren et al., 2011), adults (Vainionpaa et al., 2007; Mohr et al., 2015) and the elderly (Stolzenberg et al.,

2003) compared to controls. Sedentary individuals may respond to low-impact loading and augment bone mass or structure, whereas individuals that are more habitually active may need greater stimuli for bone gains to occur (Turner & Robling et al., 2003).

The responsiveness of the skeleton to mechanical loading varies considerably with age. The growing skeleton is superior in adapting to loads than the mature skeleton. Several studies in athletes participating in racquet sports have shown that the age at which training was initiated had a significant impact on bone adaptations (Kannus et al., 1995; Kontulainen et al., 2002). Girls who began training prior to menarche had a significantly greater (2 to 4 times) BMC and cross-sectional area (Kannus et al., 1995) in the dominant arm compared to the non-dominant arm than athletes who began their training after menarche. Using a similar population, a subsequent study also showed increased bone strength in girls who started training before or at menarche or who were peri-pubertal, but these positive effects were not shown in the post-pubertal group (Kontulainen et al., 2002). As such, childhood and early puberty may offer a unique window of opportunity for skeletal benefits.

The type, intensity, duration, frequency and importantly the magnitude of mechanical loading are key characteristics of exercise that influence bone strength and its determinants (Banfi et al., 2010). There is no agreement about the characteristics of exercise that are able to optimise bone growth and maintenance, and minimise bone loss. However, current evidence suggests that bone is most responsive to exercise that is dynamic, of high or medium impact, short in load duration and is odd or non-repetitive in load direction (Banfi et al., 2010; Weaver et al., 2016; Lombardi et al., 2015a; Marques et al., 2011).

2.7. Bone disorders

2.7.1. Osteoporosis

Osteoporosis is a common disorder of bone remodelling. It is characterised by reductions in bone mass and strength up to the point where the skeleton fails to perform its supportive function, predisposing it to fragility fractures (Kanis et al., 2013; Baron & Kneissel, 2013). Osteoporosis is considered a silent disease; as it remains asymptomatic until it is clinically manifested by fractures. Women are affected more than men; 30 to 50% of women and 15 to 30% of men will sustain an osteoporotic fracture in their lifetime (Johnell & Kanis, 2006). These rates are translated as occurrence of an osteoporotic fracture every 3 seconds, with approximately 2,000 fractures per day or 9 million fractures per annum worldwide (Akesson et al., 2013). Osteoporotic fractures at some skeletal sites (*e.g.*, hip, vertebra) are associated with greater disability, morbidity and excess mortality, including reduced function, pain, loss

of height, deformity and low quality of life (Center et al., 1999). Sustaining an osteoporotic fracture increases the risk by two to four times for subsequent fracture occurrence (Klotzbuecher et al., 2000). In addition to the devastating personal health consequences, osteoporosis increases direct (*e.g.*, hospitalisation cost) and indirect (*e.g.*, reduced productivity, equipment, long-term care) economic costs. It is estimated that osteoporotic fractures cost €37 billion each year to health care systems in Europe and approximately US $20 billion per year in the US (Hernlund et al., 2013).

The health burdens associated with osteoporosis underpin the importance of optimising bone mass and reducing fracture risk. Although prevention and treatment strategies have been historically directed towards older individuals already at risk of osteoporosis, it is now understood that the susceptibility to the disease is acquired throughout the lifespan (Harvey et al., 2014). Mathematical models suggest that the peak bone mass achieved in young adulthood is a strong predictor of fracture risk, with at least equal importance as bone loss after this point (Horsman & Burkinshaw, 1989; Hernadez et al. 2003). Thus, maximisation of peak bone mass and maintenance of adult BMD may enhance the bone reserve and contribute to the reduction in osteoporotic risk later in life (Heaney et al., 2000; Rizzoli et al., 2010).

2.7.2. Stress fracture injury

Stress fracture injuries can be categorised into insufficiency or fatigue fractures (Datir et al., 2007). Insufficiency stress fracture injuries are common in older adults and result from normal loading applied to weakened bone (Datir et al., 2007). In contrast, fatigue stress fractures commonly occur in young adults when they experience repetitive loading that the skeleton is unable to withstand (Warden et al., 2006; 2014). The repetitive nature of the loading required for stress fracture injury occurrence also differentiates them from traumatic fractures, which are single incidence events. This literature review focuses on fatigue fractures, as they are the common type of injury diagnosed in physically active populations, who are the subject of this thesis.

The pathophysiology underlying stress fracture injury is poorly understood. Two main models have been developed, the first model emphasises the role of mechanical loading, supporting the theory that repetitive mechanical loading above the bones' fracture threshold results in the accumulation of microdamage and, if unchecked, the development of a stress fracture (Warden et al., 2006; 2014; Schaffler et al., 1990). The second model suggests an interaction between mechanical loading and bone remodelling (Figure 2.7). In this model,

increased and repetitive mechanical loading increases local bone strain and initiates damage. The bone is able to target remodelling in the areas of damage, which are possibly sensed by the osteocytes (targeted remodelling, please see section 2.4). As such, the damaged area is removed by the osteoclasts (bone resorption) and refilled with new bone by the osteoblasts (bone formation). Damage repair through remodelling effectively reduces bone strain for a given load, enabling the bone to tolerate greater loads before microdamage occurrence (Warden et al., 2005). However, factors that modify the load applied to a bone and/or factors that modify the ability of bone to resist load or repair bone, may increase bone remodelling sites, thus increasing the porosity of the cortex and reducing bone mass locally (Warden et al., 2006; 2014 and Shaffler et al., 1990). Bone stiffness and strength are rapidly affected and repetitive loading in this region increases strain and may lead to the accumulation of micro-cracks and stress fracture injury (Warden et al., 2006; 2014) (Figure 2.7).

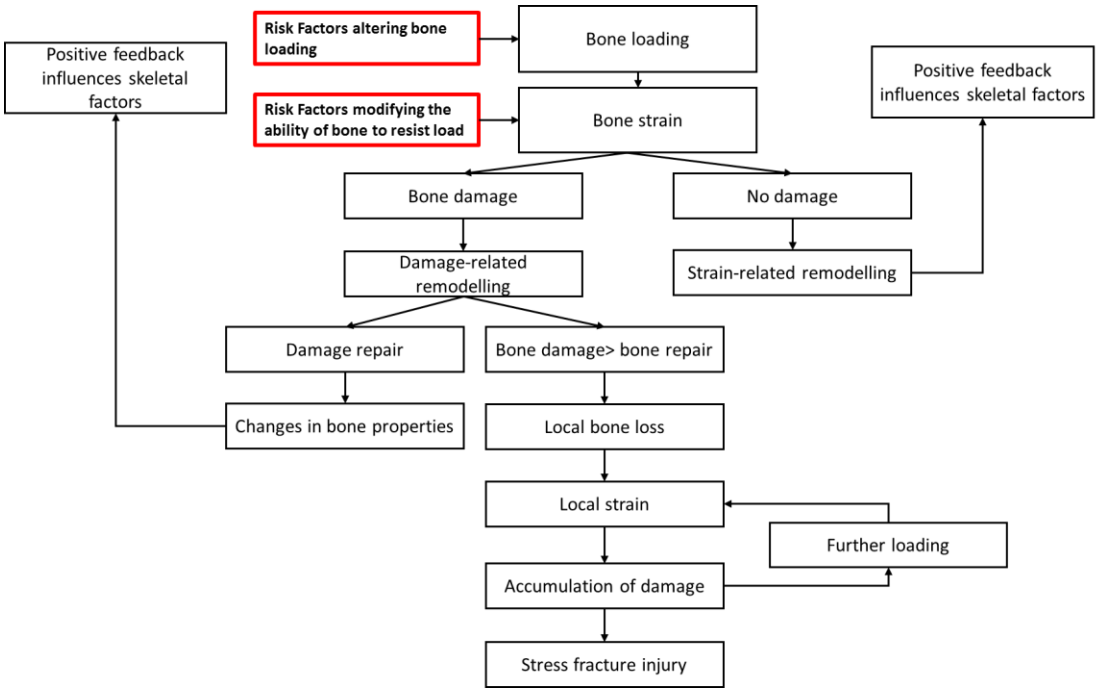


Figure 2.7. Pathophysiology for stress fracture injury (Adapted from Warden et al., 2006; 2014 and Shaffler et al., 1990).

Stress fracture injuries are one of the most common injuries in athletes (Fredericson et al., 2006; Snyder et al., 2006). The highest incidence is estimated in track and field athletes with rates between 10 and 31% (Johnson et al., 1994; Bennell et al., 1996a). In military personnel, the incidence of stress fracture ranges greatly, up to 49%, depending upon population, training programme and phase (Giladi et al., 1991; Kelly, 2000; Finestone et al., 2011; Milgrom et al., 1985). Sex differences in stress fracture epidemiology have also been

reported, with some studies demonstrating higher incidence rates in women compared to those in men (Goldberg & Pecore, 1994; Johnson et al., 1994; Wentz et al., 2011), whilst others have shown similar incidence rates between sexes (Bennell et al., 1996a; Peter and Smith, 1992; Winfield et al., 1997). Anatomical sites of stress fractures injuries are often specific to the activity performed; supporting the model of increased mechanical loading in the area of development. For example, the tibia is the most common stress fracture site in long distance runners and stress fractures of the ribs are almost unique to rowers (Bennell et al., 1996a; 1999b).

A great number of risk factors have been implicated in the pathophysiology of stress fracture injuries (Warden et al., 2006, 2014; Jones et al, 2002; Bennell et al., 1999b). These factors have been sub-grouped as factors that i) alter the load applied to a bone and ii) modify the ability of bone to resist load (Warden et al, 2014). Examples of factors that alter the load applied to bone include biomechanical factors (*e.g.,* ground reaction force magnitude and frequency, anthropometry, alignment), training factors (*e.g.,* duration, intensity and frequency of training sessions), physical fitness (*e.g.,* flexibility, muscle strength, aerobic endurance), training surface and terrain and equipment uses (*e.g.,* shoes and inserts) (Warden et al., 2014; Beck et al., 2000; Bennell et al., 1999b; Milgrom et al., 2003). Factors that modify bone resistance to loading include bone phenotypes *(e.g.,* bone mass, geometry and bone turnover), dietary factors (*e.g.*, EA, calcium, Vitamin D, disordered eating), endocrine profile (*e.g.*, oestrogen deficiency), training factors (*e.g.,* recovery time) (Warden et al., 2006; 2014; Wentz et al., 2012; Murguia et al., 1988; Nieves et al., 2010; Lappe et al., 2008). Clearly, there is no single cause of stress fracture injuries and it appears there is much overlap between risk factors. For example, exercise duration, intensity and frequency may determine the load applied to a bone. Additionally, exercise through increased EEE may contribute to low EA and alter endocrine profile; thereby, modifying the ability of bone to resist load.

2.8. EA

The concept of EA originates in ecology and biology and describes how animals use digestible energy to overcome an energetically demanding, environmental challenge (*e.g.,* cold exposure or cost of foraging) and to cover their physiological functions (Wade & Jones, 2004; Wade & Schneider, 1996). In human physiology, exercise is the environmental challenge, thus, EA defined as DEI minus EEE, represents the amount of energy accessible for use for physiological functions after taking into account the energetic demands of exercise. EA is commonly normalised to muscle tissue (kilograms of LBM or FFM) to represent the metabolically active tissue and account for individual differences in body composition (Loucks &Thuma 2003; Ihle & Loucks, 2004; Loucks, 2013). An EA of approximately 45 $kcal \cdot kgLBM^{-1} \cdot d^{-1}$ has been reported to result in energy balance and is capable of maintaining bodily functions in normal-weight healthy young adult females (Loucks & Thuma, 2003; Ihle & Loucks, 2004; Loucks, 2013). When EA drops below this level, the body uses metabolic stores and develops compensatory mechanisms to conserve energy (Loucks, 2007; 2013). Energy is prioritised for essential functions, such as cellular maintenance, thermoregulation, sensory function and locomotion; therefore, becomes unavailable for the other less essential for survival needs such as bone health, immunity and reproduction, which are compromised to various degrees depending on the extent of energy deficiency (Wade & Jones, 2004; Wade & Schneider, 1996) (Figure 2.8.).

Several populations experience low EA such as: physically active populations, through increased training volume with or without food restrictions (Loucks et al., 2007; 2011), rural populations, due to increased occupational physical activity and seasonal food unavailability (Pontzer, 2015), patients with eating disorders/disordered eating and/or hyperactivity patterns (Miller 2011; Zipfel et al., 2013), obese/overweight individuals under weight loss programmes (diet and/or exercise) (Zibellini et al., 2015; Soltani et al., 2016) and bariatric surgery patients (Scibora et al., 2012; Scibora et al., 2014). As such, different modalities and combinations of DEI and/or EE may result in reductions in EA (Figure 2.9.). In this second part of the literature review, the focus will be placed upon the concept of EA as utilised in a sports nutrition context. Furthermore, current evidence on the effects of low EA on bone metabolism and health in physically active (non-obese) populations will be synthesised.

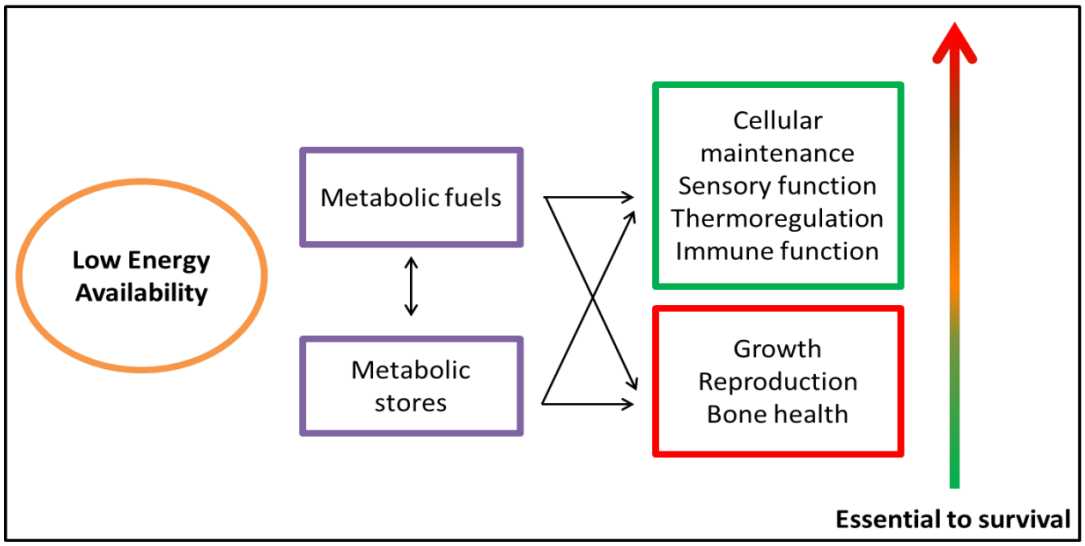


Figure 2.8. The association between low energy availability and bodily functions. Low energy availability triggers alterations in metabolic fuels and stores, which, in turn, alter the partitioning of energy. The less critical for survival functions are compromised (red box), whereas essential for survival processes are prioritised (green box). Drawn by author.

2.9. Aetiology of low EA

In physically active populations low EA can be attained intentionally or unintentionally (Loucks, 2007; 2013) (Figure 2.9). Low EA may result from intentional efforts to restrict food intake or to exercise more, with the goal to achieve or maintain a specific body weight or body composition (Nattiv et al., 2007; Loucks, 2007). Athletes participating in aesthetic sports (*e.g.*, diving, ballet, figure skating), weight category sports (*e.g.*, judo, boxing, lightweight rowing) or sports in which light weight offers gravitational advantages (*e.g.*, long distance running, cycling, cross-country skiing) are more prone to adopt disordered eating behaviours (Nattiv et al., 2007; Mountjoy et al., 2014; Sundgot-Borgen & Trosveit, 2010). Clinically diagnosed eating disorders, namely anorexia nervosa and bulimia nervosa, have been reported among physically active women, with greater prevalence reported in weight sensitive sports (42% in aesthetic sports and 24% in endurance sports) and lower prevalence rates among ball sports (Greenleaf et al., 2009; Sundgot-Borgen & Trosveit, 2004; Thieman et al., 2015) Disordered eating behaviours (e.g., binge eating, use of laxatives of diuretics) in the absence of an eating disorder diagnosis are also common among athletic individuals and may lead to low EA (Joy et al., 2016; De Souza et al., 2014a; Sundgot-Borgen & Trosveit, 2010).

Unintentional low EA may occur when individuals fail to compensate for the energy cost of exercise by analogously increasing their dietary energy intake, with this disparity being

particularly pertinent during periods of increased training volume (Stubbs et al. 2004, Loucks 2007; Loucks et al., 2011). This is unsurprising, given that some athletes train or compete in sports that contribute to high TEE, such as cycling (Tour de France, TEE: 8,054±9143 kcal·d^{-1} in men), skiing (TEE: 4,374 ±550 kcal·d^{-1} in women), running (TEE: 2,820±311 kcal·d^{-1} in women) or triathlon (ironman, TEE: 10,036±931 kcal·d^{-1} in men and 8,570±1014 kcal·d^{-1} in women) (Pontzer, 2015; Kimber et al., 2002). Several mechanisms have been proposed to explain the difficulty in matching nutritional intake with increased energy expenditure, such as an exercise-induced reduction in appetite (Loucks et al., 2007, 2011) and implementation of low energy diets characterised by a high water-content (*e.g.*, fruit and vegetables), high fibre and low fat consumption (Melin et al., 2016).

Other factors contributing to both intentional and unintentional low EA are: lack of knowledge and professional guidance during weight-control attempts (Loucks, 2007), incorrect body composition assessment resulting in unnecessary adaptations of athletes' DEI and training (Aerenhouts et al., 2015) and a pre-occupation with body image and body weight that is not related to sport performance (Wardle et al., 2006).

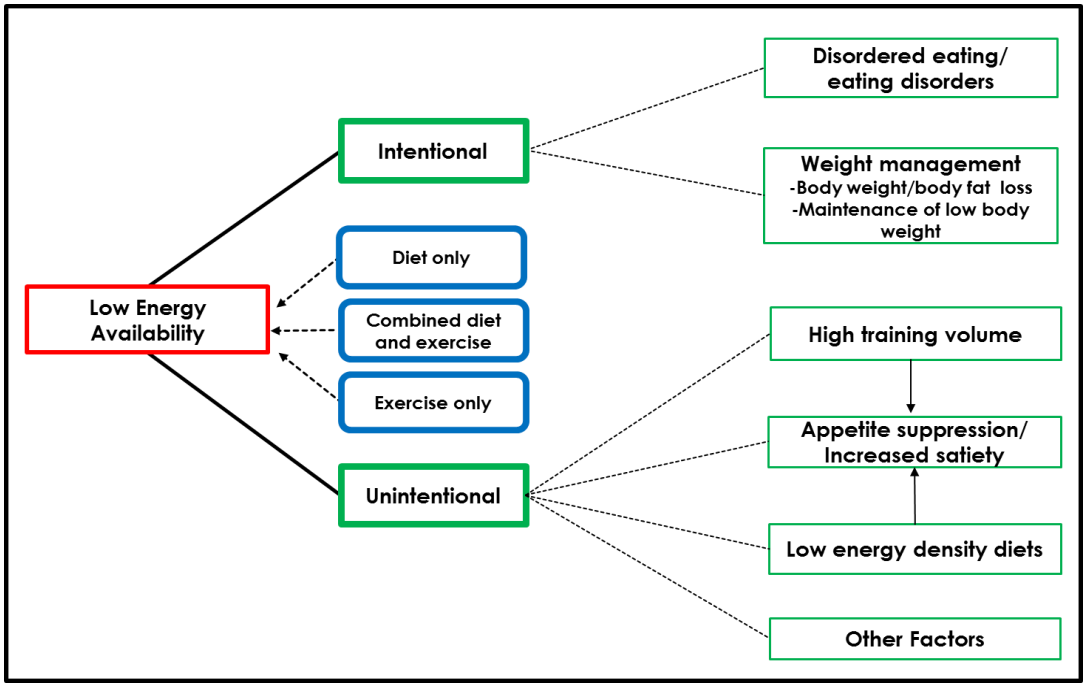


Figure 2.9. Origins (blue boxes) and causes (green boxes) of low EA in physically active individuals. Drawn by author.

2.10. EA as part of the Female Athlete Triad and the RED-S models

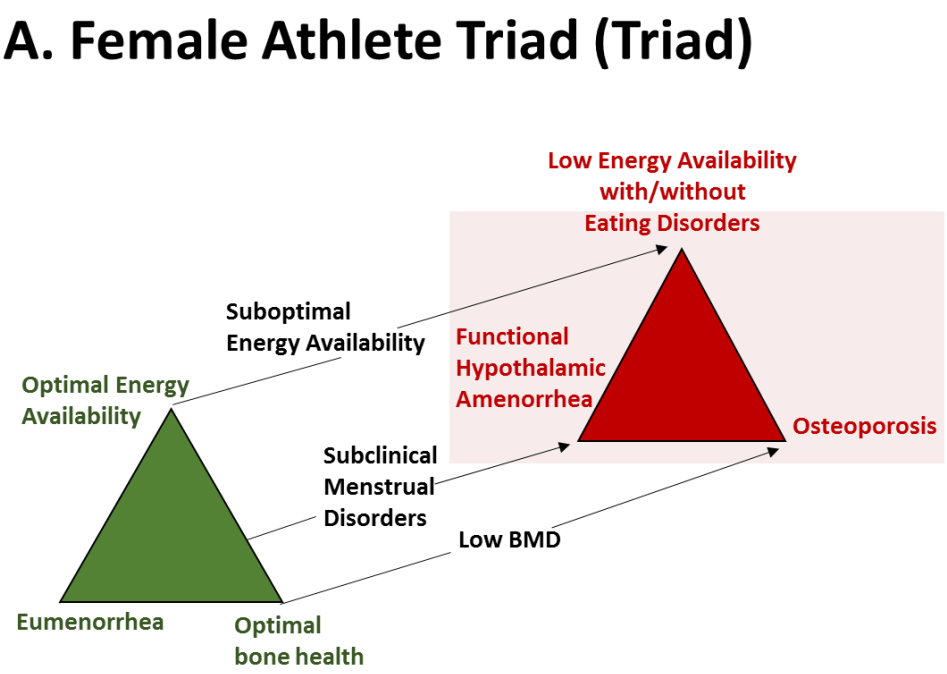
The first associations between exercise induced amenorrhea and impaired bone health were reported by Drinkwater et al. (1984) and Cann et al. (1984), who observed significantly

lower BMC among amenorrheic runners. Subsequently, Drinkwater et al. (1990) documented a significant relationship between BMD and menstrual history in female athletes (Drinkwater et al., 1990). In 1993, the American College of Sports Medicine (ACSM) created an expert group to synthesise the available evidence regarding these issues of concerns in female athletes. The first published paper in the field defined the Female Athlete Triad as the interplay between disordered eating, amenorrhea and osteoporosis in physically active girls and women (Yeager et al., 1993). In 1997, the ACSM published their first position statement on the Female Athlete Triad to provide evidence-based information about screening, diagnosis, prevention and treatment (Otis et al., 1997) (Figure 2.10.). The co-existence of all three components was a pre-requisite for the diagnosis of the Female Athlete Triad, omitting athletes at substantial disease risk (Nattiv et al., 2007; Gibbs et al., 2013). A decade later, the ACSM revised their position statement and published an updated version of the Female Athlete Triad, which developed the original static parts of the triangle into a continuum from health to disease (Nattiv et al, 2007) (Figure 2.10.). The healthy state is characterised by optimal/adequate EA (45 kcal·kgLBM^{-1}·d^{-1}), eumenorrhea and normal BMD, which is followed by suboptimal representations of EA (between 30 and 45 kcal·kgLBM^{-1}·d^{-1}), menstrual function (anovulation and luteal phase defects -please see section 2.6.1.2.) and BMD. Lastly, the clinical manifestations include low EA (<30 kcal·kgLBM^{-1}·d^{-1}) with or without eating disorders, functional hypothalamic amenorrhea and osteoporosis (the effects of low EA on bone health, within the framework of the Triad, are reviewed in more detail in section 2.11.2.). Notably, the progression of each component in the continuum occurs independently and does not necessitate the presence of the other two. This updated version of the Female Athlete Triad acknowledges the occurrence and consequences of unintentional energy deficits, as indicated by replacing disordered eating with low EA and by recognising that low EA/energy deficiency is an underlying cause of the other components. In 2014, the Female Athlete Triad Coalition published a consensus statement, which was adopted by the ACSM and the American Bone Health Alliance, with guidelines regarding treatment and management of the components of the Female Athlete Triad and the introduction of an algorithm for return-to-play of athletes with the Triad (De Souza et al., 2014a).

In 2014, the International Olympic Committee Position Stand extended the concept of the Triad, to "Relative Energy Deficiency in Sports (RED-S)", in order to broaden its definition and consequences. Thus, RED-S models extend the adverse effect of low EA on health and performance beyond those originally described in the Female Athlete Triad (Mountjoy et al., 2014) (Figure 2.10.). RED-S models propose that male athletes may also experience low EA and their subsequent adverse consequences on health and performance (Mountjoy et al.,

2014). This change in terminology has been a matter of debate, due to the paucity of studies, in women and especially in men, in many of the key areas (*e.g.*, male bone and reproductive health) proposed in the new RED-S models (De Souza et al., 2014b).

Many advances have been made in the area of EA in physically active populations. Importantly, both the Female Athlete Triad and the RED-S models support that notion that low EA triggers an unfavourable cascade of events that compromise health and performance in physically active women and men. In order to progress the RED-S paradigm, future research is required to: (i) characterise the potential for unfavourable short- and long-term effects of energy deficiency in men and (ii) explore the effects of low EA on more areas of health and performance in women.



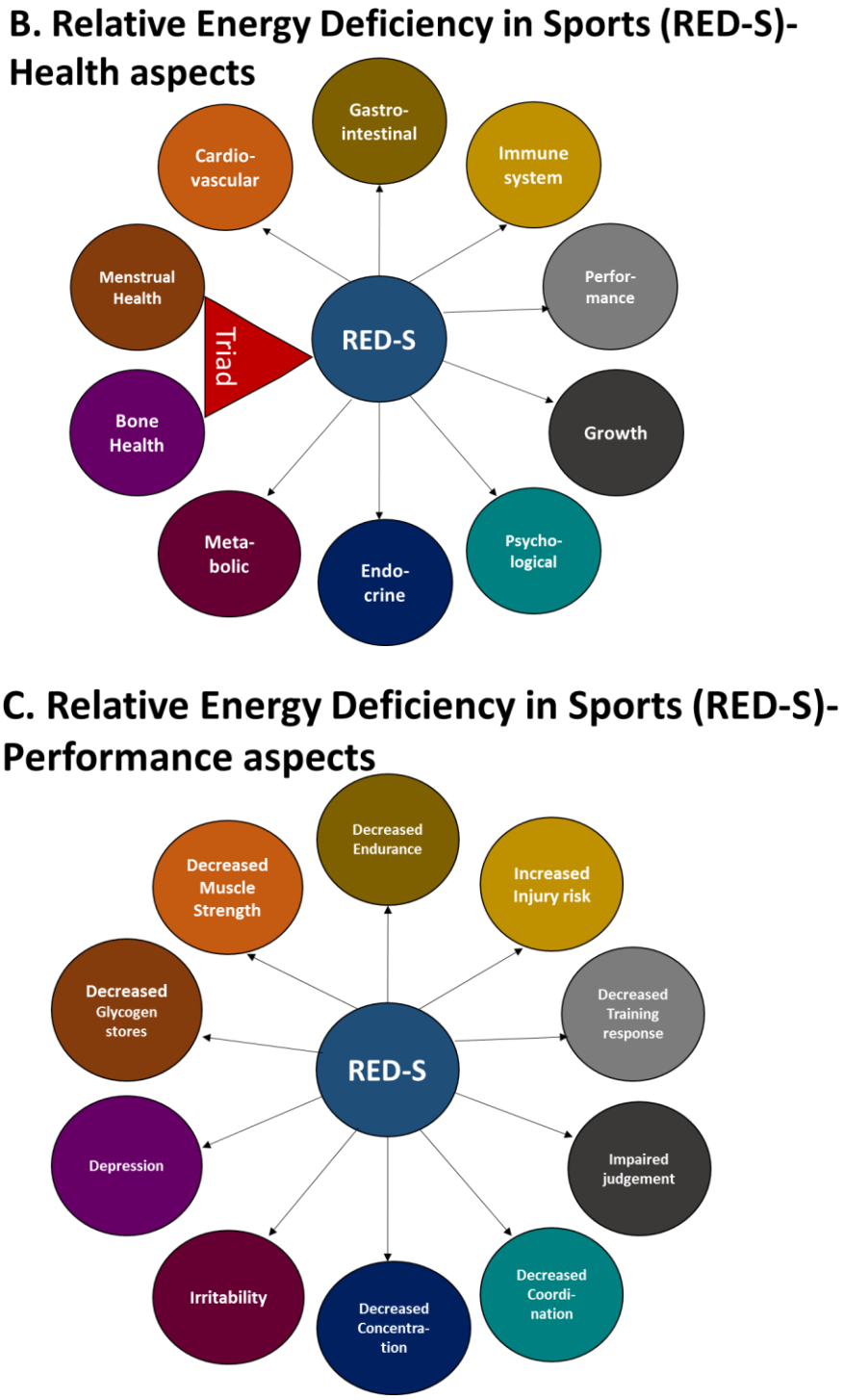


Figure 2.10. The Female Athlete Triad (Triad) (A) and the health (B) and performance (C) aspects introduced by the RED-S model. The content of the light red square illustrates the definition of the Female Athlete Triad (A) provided by Otis et al., 1997. RED-S: Relative Energy Deficiency in Sports. Adapted from Otis et al., 1997; Nattiv et al., 2007; De Souza et al., 2014a and Mountjoy et al., 2014.

2.11. Effects of energy restriction/low EA on bone metabolism and health in young, non-obese populations

2.11.1. Evidence from animal studies

The use of animal models to explore the effects of energy restriction/low EA has provided insight into the independent and synergistic effects of diet and exercise on bone metabolism and health. The similarities in the regulatory mechanisms of bone turnover between animals and humans (Frost & Jee, 1992), the accurate control of DEI and EEE, the employment of a greater number of study arms than human studies and the ability to analyse changes at a tissue-level are some of the strengths of the animal based studies (Metzger et al., 2016). In contrast, there is significant diversity among the animal models used in bone research, which requires knowledge of bone anatomy and physiology in each model and an understanding of the differences in bone parameters from those in humans (Turner, 2001b). As such, careful interpretation is required when considering the relevance of findings from animal research in relation to effects in humans.

Non-exercising animals exposed to food restriction demonstrate increased bone resorption and reduced bone formation (Talbot et al., 2001; Hamrick et al., 2008; Devlin et al., 2010), reductions in BMD (Hawkins et al., 2010; Aikawa et al., 2015) and alterations in bone microarchitecture (Talbot et al., 2001; Devlin et al., 2010; Turner & Iwaniec, 2011) and strength (Hamrick et al., 2008). Baseline body mass appears to be an important determinant of bone loss due to energy restriction, as suggested by data showing reductions in BMD (tibia, distal and proximal femur and femoral neck) and bone volume of the trabecular compartment in lean but not obese female rats following a 40% diet-induced energy deficit (Hawkins et al., 2010). Importantly, food restriction involves restrictions in both dietary energy and micronutrient intakes; the latter may also play an important role in bone conservation. Talbott et al. (1998) showed that 40% dietary energy restriction combined with 80% calcium restriction reduced BMD more than 40% dietary energy restriction alone in young female rats, suggesting that reductions in micronutrient intake may synergistically affect bone-related outcomes. Thus, studies that restrict energy and micronutrients cannot separate the individual effects of macro- and micronutrient availability and should be interpreted with this limitation in mind.

Exercise and *ad libitum* feeding increase BMD in female rats (Shiga et al., 2003; Aikawa et al., 2015). However, exercise and food reduction result in reduced EA (Hattori et al., 2013; 2014; Metzger et al., 2016) (Table 2.4.). Low bone mass (Aikawa et al., 2015; Metzger et al., 2016), low bone strength (Metzger et al., 2016) and increased resorption (Metzger et al.,

2016) have been reported at varying levels of low EA/energy deficiency achieved by combinations of exercise and diet. When compared with food restriction alone, the addition of exercise to dietary energy restriction may attenuate, but does not prevent bone loss (Swift et al., 2012; Metzger et al., 2016). Together, these findings suggest that it is important to prevent low EA, given that low EA may override the impact of increased mechanical loading provided by exercise. Future studies with robust experimental designs including magnitude of energy restriction being equal in their restricted conditions, are needed to explore how different levels of low EA and different combinations of exercise and dietary energy restriction affect bone metabolism and health.

A greater susceptibility of female versus male animals to bone loss due to reduced EA is supported by studies in female and male rats independently (Aikawa et al., 2015; Hattori et al., 2013), although no direct sex comparison has been performed in the same study. For example, 30% food restriction with or without exercise, resulted in lower bone strength and lower BMD than exercise alone or food restriction alone in female adult Sprague-Dawley rats (Aikawa et al., 2015). The same protocol applied in male adult Sprague-Dawley rats did not alter bone strength or trabecular bone volume (Hattori et al., 2013). A limitation of both studies is that exercise was prescribed on a voluntary basis and was not controlled to determine specific EEE. Thus, the restricted conditions (diet only and diet and exercise) did not result in the same level of EA. Future studies should explore potential sex differences in response to low EA on bone metabolism and health in skeletally mature animal models within the same study using well-controlled experimental conditions.

In animal models, severe food restriction disrupts the reproductive cycle and decreases gonad size in female animals (Martin et al., 2007) without affecting gonad size (Martin et al., 2007) or fertility in male animals (Nelson et al., 1997; Johnson et al., 1992). Exercising female rats, with low EA, exhibit declines in oestrogen levels with concurrent reductions in femur and tibial BMD (DiMarco, 2007). Reduced concentrations of regulatory hormones, such as IGF-1 and leptin, following low EA, occur in parallel with adverse alterations in bone-related outcomes regardless of the modality of EA/energy restriction implementation (diet-induced enengy restriction, Devlin et al., 2010; Hamrick et al., 2008; energy restriction resulting from combined dietary restrictions and exercise, Metzger et al., 2016).

Table 2.4. Summary of energy restriction/reduced EA studies through diet and exercise in animal models.

Study	Animals[1]	Duration	Groups/ conditions (exercise/food intake status)	Main results for bone-related variables
Metzger et al., 2016	Sprague-Dawley female rats 5-month	12 weeks	1.Exercise- ad libitum fed (EX-Adlib) 2.Exercise 10%- 30%ood restricted (EX-ER) 3.Sedentary-40% food restricted(SED-ER)	Areal BMD was lower after 12 weeks for EX-ER vs EX-Adlib. EX-ER Areal BMD was higher than SED-ER. EX-ER demonstrated higher cortical volumetric BMD at the midshaft tibia vs SED- ER CTX was higher for EX-ER than EX-Adlib and P1NP declined in all groups. Exercise during ER mitigated some, but not all, of the bone loss shown in SED-ER rats. EA between ER groups was equal.
Aikawa et al., 2015	Spargue-Dawley female rats 7-week old	12 weeks	1.Sedentary-ad libitum fed (SED-Adlib) 2.Voluntary exercise-ad libitum fed (EX-Adlib) 3.Sedentary-30% food restricted (SED-FR) 4.Voluntary exercise -30% food restricted (EX-FR)	The interaction of exercise and food restriction (EX-FR) resulted in, lower bone strength and lower BMD (lumbar spine; total, proximal metaphysis and diaphysis tibia) than exercise alone (EX-Adlib) or food restriction alone (SED-FR). EA was lower in EX-FR than SED-FR, which may have contributed to these results.
Hattori et al., 2014	Sprague-Dawley male rats 4-week	13 weeks	1.Sedentary-ad libitum fed (SED-Adlib) 2.Voluntary exercise-ad libitum fed (EX-Adlib) 3.Sedentary-30% food restricted (SED-FR) 4.Voluntary exercise -30% food restricted (EX-FR)	Reductions in femur bone strength, tibial BMD, trabecular and cortical bone volume were shown in EX-FR group compared to SED-Adlib. These results suggest that food restriction causes low bone strength and deterioration in bone architecture in exercising growing male rats.
Hattori et al., 2013	Spargue-Dawley male rats 14-week old	13 weeks	1.Sedentary-ad libitum fed (SED-Adlib) 2.Voluntary exercise-ad libitum fed (EX-Adlib) 3.Sedentary-30%food restricted (SED-FR) 4.Voluntary exercise -30% food restricted (EX-FR)	The EX-FR, SED-FR and EX groups did not differ in trabecular bone morphology and strength from SED. Cortical bone volume decreased in EX-FR compared to EX. These results suggest that low EA has little effect on bone tissues in adult male rats. No information if restricted conditions resulted in similar reduced EA- exercise and food intake data suggest a lower EA in EX-FR compared to SED-FR.
Swift et al., 2012	Sprague-Dawley female rats 4-month	12 weeks	1.Sedentary- ad libitum fed (SED-Adlib) 2.Exercise- ad libitum fed (EX-Adlib) 3.Exercise-40% calcium restriction only (EX-CaR) 4. Energy restricted (40%) though exercise (EX-ER) 5. Exercise-40% food restricted (EX-FR)	Total body BMD were lower in EX-ER and EX-FR rats compared with the EX-Adlib. EX-CaR had few negative effects on bone. Declines in total volumetric BMD, at the proximal tibia metaphysis were shown in EX-ER and EX- FR groups. EX-FR exhibited increased osteoclast surface and decreased mineral apposition rate in cancellous bone. Exercise attenuated some, but not all, deleterious effects on bone after energy or food restriction but is more protective during Ca restriction.

[1]Animals (animal model, sex, age); EX: Exercise; SED: Sedentary; Adlib: *ad libitum; FR:* food restricted; CaR: calcium restriction; CTX: C-terminal telopeptides of type I collagen; P1NP: Amino-terminal Pro-peptides of Type 1 Procollagen

2.11.2. Evidence from human studies –the Female Athlete Triad and the RED-S models

EA is the cornerstone component of the Female Athlete Triad and the RED-S models and underpins the pathophysiology of the remaining components (De Souza et al. 2014a; Mountjoy et al., 2014; 2015). EA affects menstrual function and bone health independently. Menstrual disturbances associated with low EA may also unfavourably influence bone health (Mallinson & De Souza, 2014; De Souza et al., 2008; 2014a).

2.11.2.1. BMD

In the Female Athlete Triad, low BMD is defined as a Z-score < -1.0 (in particular, between -1.0 and -2.0) in the presence of additional clinical factors (Nattiv et al., 2007), rather than T scores<-2.5 used for the diagnosis of osteoporosis by the World Health Organisation (Kanis et al., 2013). More specifically, a T-score is defined as the number of standard deviations over or below the mean BMD of a healthy, adult, reference population. For men younger than 50 years and premenopausal women the use of Z-score has been recommended (Kanis et al., 2013). This is defined similarly to T-score but uses an age-matched healthy population for comparison (Kanis et al., 2013). This is, at least in part, due to the fact that athletes participating in weight bearing sports have 5-15% greater BMD than non-athletes (Nattiv et al., 2007). In a systematic review, Gibbs et al. (2013) showed that the prevalence of low BMD varied between 0-15.4% when defined as Z-score ≤ -2.0 and by 0 to 39.8% when defined as Z-score between -1.0 to -2.0. Numerous reports have shown that amenorrheic athletes, who experience energy deficiency and hypogonadism, have lower BMD than eumenorrheic controls, especially at sites with greater contribution of trabecular bone (Zanker & Swaine, 1998a; Cobb et al., 2003; Christo et al., 2008; Melin et al., 2015). Links between disordered eating and low BMD have also been established in physically active females with or without menstrual disturbances (Cobb et al., 2003; Barrack et al., 2008; Rauh et al., 2010; Melin et al., 2015). There is little evidence available documenting the prevalence of low EA and BMD. In their systematic review, Gibbs et al (2013) included studies published between 1975 and 2011 that reported the prevalence of the Female Athlete Triad. From these studies, only one report by Hoch et al., 2009 included the components of the Triad according to the 2007 ACSM position stand (please see section 2.10-Nattiv et al., 2007; De Souza et al., 2014). Low EA (<45 kcal·kgLBM^{-1}·d^{-1}) and low BMD were prevalent in 36 and 13% of high school female athletes respectively, with EA calculations based on survey information (Hoch et al., 2009). There is still paucity of studies that have measured low EA and BMD concurrently in physically active females (Robbeson et al., 2013; Goodwin et al., 2014; Melin et al., 2015). Melin et al., (2015) examined the prevalence of the Female Athlete Triad-associated conditions in 40 endurance female athletes; 42.5% had

suboptimal EA (30≤ EA (kcal·kgLBM^{-1}·d^{-1}) ≤ 45) and 20% demonstrated low EA (<30 kcal·kgLBM^{-1}·d^{-1}). Although there was no significant association between EA and BMD; out of the 8 participants with low EA, five had normal BMD, one had a BMD Z-score between -1.0 and -2.0 and two participants had a BMD Z-score<-2.0 (Melin et al., 2015). Similarly, in a population of Kenyan female runners, low EA (<45 kcal·kgLBM^{-1}·d^{-1}) and low BMD were independently prevalent in 92% and 76% of the athletes and their concurrent presence reached a prevalence rate of 56% (Goodwin et al., 2014). In a longitudinal study, Viner et al (2015) measured how EA varied across the competitive season in a mixed population of male and female competitive endurance cyclists with lower than expected BMD (Z score< 0). They showed that 70% of participants had low EA <30 kcal·kg FFM^{-1}·d^{-1} during pre-season, 90% during competition and 80% during off-season; whilst there were no significant sex differences (Viner et al, 2015). Although these findings suggest that low EA and BMD co-exist in physically active women, the cross-sectional study design does not allow the extrapolation of a cause effect relationship between low EA and low BMD. The comparison of prevalence rates of low EA and BMD reported in athletes partaking in differerent sports is challenging mainly due to the discrepancies in the definitions used for low EA and low BMD and the variability in the methods utilised to determine EA (DEI, EEE, body composition). For example, DEI has been assessed by using food records (Melin et al., 2015), 24-h recalls (Robbeson et al., 2013), surveys (Hoch et al., 2009), pictures of food and drink items before and immediately after consumption (Shaal et al., 2016), with these methods differing greatly in accuracy (Magkos & Yiannakoulia, 2003) and introducing variability in EA values.

Bone health studies in athletic men are scarce and limited by small sample sizes, unclear definitions of low BMD and low EA cut-offs and have failed to synchronously capture EA and BMD. Male athletes who partake in weight-sensitive sports and/or non-weight bearing activities are at risk for low BMD (Mountjoy et al., 2014). For example, low BMD values have been reported in male jockeys (Dolan et al., 2012; Warrington et al., 2009; Wilson et al., 2013), with the authors attributing these findings to jockeys' rapid and extreme weight management practices. These findings reinforce the significantly lower BMD Z-scores seen among flat jockeys who compete at low BMI and possibly experience severe energy deficiency (Wilson et al., 2014). Disordered eating (Tenforde et al., 2015) and volume of endurance training (indicative of high EEE) (Hind et al., 2006; Kemmler et al., 2006) contribute to low EA and have been associated with BMD deficits in male athletes. Non-weight bearing exercise may exacerbate the effects of low EA on BMD. EAs as low as 7 kcal·kg FFM^{-1}·d^{-1} (Vogt et al. 2005) have been shown in male cyclists during periods of high training volume and low BMD (lumbar spine) is a consistent finding among male

cyclists (Olmedillas et al., 2012; Nichols et al., 2010; Rector et al., 2008). Conversely, high impact exercise provides an osteogenic stimulus that may counteract some of the negative effects of low EA, with apparent effects at weight bearing sites in athletic males. This notion is supported by Dolan et al. (2011) who compared boxers and jockeys, as they engage in similar weight loss practices, and showed higher BMD among the boxers. Similarly, Tenforde & Fredericson (2011) showed greater regional BMDs in male soccer players compared to runners, and sedentary controls. Endurance runners who may experience low EA (Tenforde et al., 2016; Mountjoy et al., 2014) have higher BMD at loaded sites only compared to controls (Tenforde & Fredericson, 2011; Hetland et al., 1993; Hind et al., 2006). Taken together, current evidence indicates that male athletes with low EA are more likely to have low BMD values, which is similar to low BMD seen among their female counterparts.

2.11.2.2. Bone geometry, microarchitecture and strength

An increasing number of studies are using pQCT and HR-pQCT to assess bone geometry, microarchitecture and strength in the area of the Female Athlete Triad (Ackerman 2011; 2012a; Mitchell et al., 2015). Adolescent and young adult amenorrheic athletes appear to benefit from the osteogenic effects of exercise at weight bearing sites associated with habitual exercise; they exhibit similar total bone area (bone size), trabecular area and cortical perimeter at the tibia compared to eumenorrheic athletes and greater values than those of non-athletic controls. They have, however, lower trabecular number, greater trabecular separation and lower ratio (%) of cortical to total area, together with lower cortical density at the weight bearing tibia (Ackerman et al., 2011; 2012a). These data suggest a larger bone size resulting from exercise at loaded sites with reduced cortical thickness perhaps due to endocortical resorption in the presence of low oestrogen levels in amenorrheic athletes (Ackerman et al., 2011). At the non-weight bearing radius, total and trabecular volumetic BMD are lowest in the amenorrheic athletes (Ackerman et al., 2011; 2012a). Application of finite element analysis to the HR-pQCT images (please see section 2.5.2.) showed that estimated tibial stiffness and failure load were higher in the eumenorrheic athletes than in the non-athletes. This weight-bearing benefit is absent in the amenorrheic athletes, as suggested by the non- significant difference in stiffness and failure load between the amenorrheic athletes and the controls. At the non-weight bearing radius, failure load and stiffness were significantly lower in the amenorrheic athletes than in the non-athletes (Ackerman et al., 2012a). By using individual trabecula segmentation (please see section 2.5.2.), a recent study further supported these results showing that trabecular morphology and alignment differed among amenorrheic and eumenorrheic athletes and non-athletes, with

these differences potentially related to a higher fracture risk in amenorrheic athletes (Mitchell et al., 2015). Although menstrual status is an indicator of low EA, the aforementioned studies did not determine the EA status in this population. The exploration of bone microarchitecture and strength in men with RED-S is currently lacking.

2.11.2.3. Risk for stress fracture injury

Barrack et al. (2014) explored several Female Athlete Triad-related factors in association with the development of stress fracture injuries in a large sample of female athletes. Low BMD (*Z*-score <−1.0), participation in >12 hours of purposeful exercise per week, and a BMI<21.0 $kg \cdot m^{-2}$, were more commonly seen among female athletes who had sustained a stress fracture injury (14.7%-21.0%) compared to fracture-free athletes (3.4%-7.6%) (Barrack et al., 2014). Furthermore, the investigators demonstrated a dose-response relationship between Female Athlete Triad-related factors and stress fracture injury; female athletes who presented with any of these three Female Athlete Triad-related factors were 2.4-4.9 times more likely to sustain a stress fracture, and the concurrent presence of all three increased the risk of injury by 6.8-fold (Barrrack et al., 2014). Menstrual disturbances have been consistently associated with stress fracture injury (Duckham et al., 2012; S; Cline et al., 1998) and may contribute to more severe stress fracture injuries (Nattiv et al., 2013). This is supported by a study by Nattiv et al. (2013), where female athletes with oligomenorrhea and amenorrhea had more severe stress fracture injuries (greater MRI grades) compared to their eumenorrheic counterparts.

2.11.2.4. BTMs

Previous research has shown that athletes who may experience low EA have either increased (Hetland et al., 1993; Dolan et al., 2012; Barrack et al., 2010) or decreased bone turnover (Brahm et al., 1997; Zanker & Swaine, 1998b), as evidenced by a synchronous increase in bone formation and resorption or a parallel decrease in both. Mean BTM levels have been shown to be towards the higher end of clinical ranges in jockeys (Wilson et al., 2013; Wardon-Lynch et al., 2010) and endurance runners (Hetland et al., 1993; Barrack et al., 2010). In contrast with these findings, reduced bone formation and resorption were reported in amenorrheic female athletes compared with oligomenorrhoeic or eumenorrhoeic athletes (Zanker & Swaine, 1998b) and in male runners compared to age-matched controls (Brahm et al., 1997). The clinical significance of these findings is unclear, since either increased or decreased bone turnover may accompany changes in bone properties (Davison et al., 2006; Hernadez, 2008; Bouxsein, 2005). Increased bone turnover decreases the duration of secondary mineralisation leading to under-mineralised bone (Davison et al., 2006), and

modifies the isomerisation of collagen (Viguet-Carrin et al., 2006). Conversely, suppressed bone turnover may impair bone-repairing capacity leading to microdamage accumulation and altered mechanical properties (Bouxsein 2005; Davidon et al., 2006).

De Souza et al. (2008) used four experimental conditions (energy and oestrogen replete, energy replete oestrogen deplete, energy deplete oestrogen replete and energy and oestrogen deplete) to examine the independent and synergistic effects of energy and oestrogens on BTMs in exercising women. The energy replete groups showed no changes in either bone formation or resorption independent of oestrogen status. Energy and oestrogen deficiency resulted in significantly reduced bone formation (P1NP) and elevated bone resorption (urinary β-CTX) (De Souza et al., 2008) compared to the levels of these markers in the other groups. This was accompanied by the lowest BMD values in the energy and oestrogen deficient group. This study suggests an uncoupling of bone turnover that resulted in changes in BMD, when individuals were deficient in both energy and oestrogen. These finding should, however, be interpreted cautiously. The selection of β-CTX measured in urine as a marker of bone resorption is surprising, given the requirements for creatinine adjustments and standardisation of urine sample collection (Vasikaran et al., 2011; Lombardi et a., 2012a). Furthermore, this study was observational and did not induce the oestrogen or energy deficiencies. As such, a cause-effect relationship cannot be determined and prospective studies are required to shed light on the BTM responses to low EA, and to investigate how these might translate to long-term changes in bone mass and architecture in physically active populations. Direct assessment of the nutritional and reproductive status of the study population is an important methodological consideration for future research.

2.11.3. Interventional studies in humans

A limited number of experimental studies have investigated the short and long-term effects of low EA (or energy restriction) on bone turnover and/or BMD in non-obese populations (Table 2.6.). Grinspoon et al. (1995) showed that acute fasting (without exercise) reduced bone formation (P1CP: -45%; OC: -58%) and increased bone resorption (PYD: -49% and DPD: -50%) following 4 days of energy restriction in sedentary eumenorheic women. Although the BTM measured are not included in the recommended BTM of current reference standards (Vasikaran et al., 2011, please also see section 2.5.4.5.) and earlier assays used to measure these markers were resulting in more heterogeneous results, the authors of this study suggest an uncoupling of BTM favouring resorption following 4 days of fasting. Energy restriction through exercise (60 min running) and 50% restriction of estimated dietary energy requirements reduced bone formation (P1NP), but did not alter

bone resorption (uninary NTX) in trained men (Zanker and Swaine, 2000). P1NP is reflective of bone formation, has low intaindividual variability and satisfactory assay precision (Vasikaran et al., 2011); however, urinary NTX is limited in part due to sources of errors introduced by urine samples and heterogeneous nature of NTX fragments (Vasikaran et al., 2011; Wheater et al., 2013). In this study energy restriction was determined using energy balance rather than EA. The estimation of energy balance (DEI, RMR, thermic effect of food and EEE) may involve greater uncertainty compared to EA (DEI, EEE) (Loucks 2007; 2013). This study is the only one conducted in a physically active population so far, but poor methodological rigour necessitates the conduction of further studies with tight experimental control. In subsequent, short-term laboratory studies, Loucks et al. (2004) explored the effects of three distinct levels of reduced EA at 30, 20 and 10 kcal·kgLBM^{-1}·d^{-1}on BTM in sedentary, eumenorrheic women. All three levels of reduced EA were achieved by a combination of dietary energy restriction (45, 35 and 25 kcal·kgLBM^{-1}·d^{-1}) and exercise (contributing to EEE of 15 kcal·kgLBM^{-1}·d^{-1} at 70% of their maximal oxygen uptake (VO_{2max})). This study showed a dose response relationship between reduced EA and BTM; bone formation (P1CP and OC) was suppressed at an EA below 30 kcal·kgLBM^{-1}·d^{-1}, whereas bone resorption (urinary NTX) only increased in the severely restricted EA condition at 10 kcal·kgLBM^{-1}·d^{-1} (Ihle & Loucks, 2004).

Available evidence suggests that bone formation and resorption may respond differently to changes in energy status, with bone formation being reduced first and contributing to an imbalance of bone remodelling (Zanker & Swaine, 2000; Ihle & Loucks, 2004). Only a limited number of short-term interventional studies have been conducted in the area of energy deficiency and bone metabolism, with a number of limitations in their experimental design, making it difficult to draw firm conclusions. Although EA has been identified as the underlying factor of the well-established Female Athlete Triad (Nattiv et al., 2007) and the recent RED-S models (Mountjoy et al., 2014) in physically active women and possibly men, no short-term experimental study has been conducted in the area after 2004. Out of the three available studies (Grinspoon et al., 1995; Zanker & Swaine, 2000; Ihle & Loucks, 2004), only one has been based on EA (Ihle & Loucks, 2004) and only one has been conducted in physically active populations. Thus, there is a knowledge gap pertaining to the effects of low EA on bone metabolism and health in physically active women and men.

A small number of longer-term studies have been conducted in normal weight and overweight, sedentary men and women (Table 2.6.), but not in physically active populations. Two randomised controlled trials conducted in overweight women (BMI between 25 and 30 kg·m^{-2}) demonstrated no significant BMD alterations following weight loss achieved

through dietary energy restriction alone (Riedt et al., 2007; Redman et al, 2008) or dietary energy restriction and exercise (Redman et al., 2008). These findings suggest that BMD is maintained during weight loss in young, premenopausal women. Villareal et al (2006) examined the effects of weight loss, through dietary energy restriction or increased exercise energy expenditure independently, on BTM and BMD for 1 year. Both bone formation and resorption increased independent of modality of restriction at the 6-month follow-up and translated into significant reductions up to -2.2% in total hip, intertrochanter, and spine at the 1-year follow-up when energy restriction was attained through diet, but not through exercise (Villareal et al., 2006). These findings suggest potential bone sparing effects when weight loss is exercise-induced. In a larger randomised controlled trial, the same investigators compared the effects of caloric restriction on BTM and BMD over 2 years. At 12 months, the caloric restriction group had greater increases in bone resorption (β-CTX and TRACP5b) and some decreases in bone formation (BALP, but not P1NP) than the controls. At 2 years, the caloric restriction group experienced a 2% reduction in lumbar spine, total hip and femoral neck BMD and these changes were greater compared to the changes experienced by the control group. Taken together, these studies indicate that diet-induced weight loss results in reduced BMD, which is further supported by early BTM responses. Importantly, these results are derived from a mixed (men and women), middle-aged (age; mean: 39 y, range: 20-50 y), non-obese (including some normal weight, but overall, overweight individuals; mean BMI: 27 $kg \cdot m^{-2}$) sedentary population, with some women potentially being peri- or post-menopausal. Thus, they may not be indicative of BTM and BMD responses to energy deprivation in younger, lean, physically active premenopausal women and men. Further research is warranted to explore the long-term effects of EA in these populations.

Table 2.6. Experimental studies exploring the short- and long-term effects of energy restriction (designed based on energy balance or EA concept) on bone metabolism health in non-obese participants.

Study	Participants[1]	Design	Prescrption[2]	EA[2]	Main bone related outcomes and conclusions
Short-term studies (<7 days)					
Grinspoon et al, 1995	Sedentary eumenorrheic women, A=24 y, n=14	Initial part of RCT[3] 4 days	Fasting (-100%) D	No	Fasting resulted in reductions in P1CP (-58%) and OC (-45%) and increases in PYD (+49%) and DPD (+50%).
Zanker & Swaine, 2000	Physically active men, A= 25 y, n=8	Crossover3 days	Control (0%) D+EX RES (-50%) D+EX	No	P1NP reduced (-17%) in RES only; but BALP and uNTX did not change in response to either CON or RES.
Ihle & Loucks 2004	Sedentary eumenorrheic women, A= n=14	RCT Crossover5 days	Control (0%) D+EX RES_1 (-33%) D+EX RES_2 (-66%) D+EX RES_3 (-78%) D+EX	45 30 20 10	RES_1 OC: -11%, P1CP: -12% RES_2 OC: -32%, P1CP: -19% RES_3 uNTX: +34%, OC: -28%, P1CP: -26% OC and PICP were suppressed by all restricted EA treatments (all $P<0.05$), whereas NTX only at severely reduced EA (RES_3).

Longer-term studies (<7 days) (continue)					
Villareal et al., 2006	Sedentary women and men A=57 y, n=48	RCT 1 year	Control (0) D EX (-20%) EX CR (-20%) D	No	Compared with controls, the CR group had decreases in BMD at the total hip (−2.2% ± 3.1% vs 1.2% ± 2.1%) and intertrochanter (−2.1% ± 3.4% vs 1.7 ± 2.8%). The CR group had a decrease in spine BMD (−2.2% ± 3.3%). Despite weight loss, the EX group did not exhibit any decrease in BMD. Bone turnover increased in both CR and EX.
Villareal et al., 2016	Sedentary non-obese men and women , A=39, n=218	RCT 2 years	Control (0%) D CR (-25%) D	No	Compared with the control group, the CR group had greater changes in BMD at 24 months: lumbar spine, total hip and femoral neck. Changes in BTM were greater at 12 months for β-CTX, TRACP5b and BALP but not P1NP; at 24 months, only BALP differed between groups.
Redman et al., 2008	Sedentary overweight men and women, A=38 y, n=49	RCT 6 months	Control (0%) D CR (-25%) D CR + EX (-25%) D+ EX LCD (890 kcal-approximately -50%) D	No	Compared with the control group, none of the groups showed change in total body or hip BMD. β-CTX was increased in all 3 intervention groups, with the largest change observed in the LCD group.. BALP decreased in the CR group but was unchanged in the CR + EX, LCD, and control groups. Moderate CR, with or without exercise for 6 months does not result in significant bone loss in young adults.
Riedt et al., 2007	Sedentary, overweight women, A=38 y, n=44	RCT 6 monts	CR (30%) D CR+Ca (30%) D	No	No significant decrease in BMD or rise in bone turnover was shown with weight loss at normal or high calcium intake.

[1]Participants (training status, sex, age, number); [2]Prescription (training status, sex, age, number); [3]EA concept included in the experimental design (yes-level of EA or no); [4]No control group; D: Diet, EX: Exercise; RCT: Randomised control trial; RES: Restricted; CR: Caloric restriction; EA: Energy availability; LCD: Low calorie diet; Ca: Calcium; BMD: Bone mineral density; P1NP: Amino-terminal pro-peptides of type 1 procollagen; BALP: Bone specific alkaline phosphatase; CTX: C-terminal cross-linked telopeptide of type I collagen; OC: Osteocalcin; TRACP5b: Tartate-resistant acid phosphatase 5b.

2.12. Endocrine alterations in response to low EA and bone metabolism and health

Insulin, cortisol, GH/IGF-1, triiodothyronine (T_3), leptin, ghrelin, peptide YY (PYY), glucagon-like- peptide 1 and 2 (GLP-1 and 2) are the primary hormones that are affected in response to changes in energy status (Misra & Klibanksi, 2011; 2014; Shapses & Sukumar, 2012; Walsh & Henriksen, 2010). It is important to note that most of the available evidence is coming from studies in individuals with chronic energy deficiency (*i.e.*, anorexia nervosa or weight loss efforts in overweight and obese populations, which may differ from responses to short-term EA in normal-weight, physically active individuals with normal physiological functions. Although the actions of these regulatory hormones on bone have been previously characterised, there are limited data on whether changes in their concentrations in response to EA mediate the effects of low EA on bone metabolism and health (Ihle & Loucks, 2004).

2.12.1. Insulin

Insulin, an anabolic hormone synthesised and secreted by beta pancreatic cells, regulates glucose uptake from the bloodstream to several tissues including the liver, skeletal muscle and adipose tissue. Insulin and glucose decrease in low energy states (Misra, 2012), with levels being lower in amenorrheic (Laughlin & Lin, 1996) and oligomenorrheic (Rickenlund et al., 2004) athletes and athletes with luteal phase defects (De Souza et al., 2003) compared with eumenorrheic athletes and non-athletes. Insulin receptors are present on both osteoblasts and osteoclasts (Pun et al., 1989). *In vivo*, insulin increases bone formation (insulin injection; Cornish et al., 1996) and decreases bone resorption (endogenous and exogenous stimulation tests; Bjarnasson et al., 2002). The insulin deficiency caused by energy deficiency in anorexia nervosa is accompanied by reduced BMD and increased fragility risk (Misra et al., 2007). At moderately reduced EA, reductions in bone formation are reduced in parallel with decreases in insulin levels (Ihle & Loucks., 2004) in sedentary women; however, it remains unknown if this holds true for physically active women and men.

2.12.2. Cortisol

Given that cortisol promotes gluconeogenesis, the hypercortisolaemia developed in low EA states, including anorexia nervosa (Lawson, et al. 2009; Misra, et al. 2004) and exercise-induced amenorrhea (Ackerman et al., 2013) is an established adaptation to maintain blood glucose levels (Misra, 2012). Elevated cortisol levels result in decreased bone turnover, an increased number of osteoclasts, and decreased osteoclast apoptosis, all favouring bone resorption (Canalis et al, 2007; Mazziotti et al., 2015). Impairments in bone formation have also been reported with reductions in osteoblasts number, a shift in progenitor cells from

osteoblasts to adipocytes, and accelerated osteoblast apoptosis (Canalis et al., 2007). Some indirect effects of cortisol may be mediated though the inhibition of GH (and thus, IGF-1) and gonadotropins or an increase in PTH receptor expression on osteoblasts, which have an anabolic effect on bone (Misra & Klibanski, 2014). Negative correlations have been shown between cortisol concentrations and bone formation (Misra et al., 2004), and cortisol concentrations and BMD (Lawson et al. 2009) in anorexia nervosa and hypothalamic amenorrhea, but such relationships have not been investigated in the short-term in physically active populations.

2.12.3. GH/IGF-1

The GH/IGF axis is affected by both acute and chronic energy deficiency (Grinspoon et al., 1995; 1996; Misra & Klibanski, 2014). GH release reaches its peak at night with this peak being exaggerated by fasting and supressed by feeding (Walsh & Henriksen, 2010). In response to chronic, severe energy restriction, increased GH concentrations and GH resistance have been reported (Misra & Klibanski, 2011). GH has anabolic effects on bone; it directly stimulates the proliferation and differentiation of osteoblast precursors through GH-receptors present on osteoblasts (Giustina et al., 2008). GH exerts indirect effects on bone mediated through IGF-1 (GIustina et al., 2008). IGF-1 reductions have been reported in adults under energy or protein restriction (Fontana et al. 2008; Zanker & Swaine, 2000; Grinspoon et al., 1995). In the short-term, suppression of IGF-1 may be masked by increases in circulating concentrations of binding proteins (Fontana et al., 2016); therefore, it is useful to determine the levels of binding proteins. IGF-I has well-established anabolic effects on bone; it regulates osteoblasts' activation either directly by stimulating IGF-1 receptors or indirectly via RANKL-synthesis by osteoblasts (Giustina et al., 2008). IGF-I also promotes bone formation by stimulating proliferation and differentiation of chondrocytes and osteoblasts and increasing the synthesis of type I collagen (Giustina et al., 2008; Mochizuki et al., 1992). Low IGF-I levels have been associated with reductions in P1NP during short term energy restriction in physically active males (Zanker & Swaine, 2000), with reduced BMD (Liu et al., 2008) and unfavourable changes in parameters of bone microarchitecture in women under energy deprivation (Faje et al. 2013; Lawson et al. 2010).

2.12.4. Leptin

Leptin is the protein product of the obese or ob gene and acts as a peripheral signal to the brain to convey information about the amount of energy available in adipose tissue and/or changes in EA (Chan & Mantzoros, 2005; Upadhayay et al., 2015). Thus, leptin has a major role in the regulation of energy balance and also acts as a physiological cue that links energy

status with reproduction (Donato et al., 2010), bone metabolism (Upadhyay et al., 2015) immunity (Conde et al., 2010), wound healing (Schreml et al., 2010) and cardiovascular function (Sweeney, 2010). Reduced leptin levels have been shown in anorexia nervosa (Grinspoon et al., 1996) and highly trained athletes with low body fat stores (Thong et al., 2000). In animal models, centrally administered leptin inhibits bone formation and induces bone loss (Ducy, et al. 2000), whereas peripheral leptin exerts anabolic effects on bone and reduces bone fragility in leptin-deficient animals (Cornish et al., 2002; Hamrick, et al. 2008). Leptin may exert its bone effect directly through its receptors on osteoblasts and chondrocytes, by activating fibroblast growth factor 23 (FGF-23) (bone trophic factor) and regulating OC (Upadhayay et al., 2015). Leptin may also have local effects on bone, as bone marrow adipocytes have been shown to secrete leptin. Indirectly, leptin may alter other hormones including oestrogen, cortisol, IGF-1 and PTH that may, in turn, mediate bone responses (Upadhayay et al., 2015). Indeed, in humans, metreleptin replacement therapy in women with hypothalamic amenorrhea results in increased LH, oestrogen, IGF-1 and thyroid hormones levels, all of which favourably impact bone health (Welt et al., 2004). The effects of low EA on leptin levels in physically active men and women need to be elucidated and characterised in relation to alterations in bone metabolism. The exploration of sex differences is also of interest given that men and women differ in the amount of body fat upon which leptin concentrations depend (Chan & Mantzoros, 2005).

2.12.4. T_3

The thyroid hormones are important mediators of energy and bone metabolism (Lakatos et al., 2003; Bassett & Williams, 2008). T_3 is one of the most active forms of thyroid hormone, which is converted from thyroxine (T_4) in the peripheral tissues (Bassett & Williams, 2008). T_3 has been suggested as an indicator of energy deficiency, as decreases in T_3 induce adaptive mechanisms to conserve energy in underweight individuals (Misra & Klibanski, 2011) and in sedentary, eumenorrheic women exposed to low EA (Loucks & Callister, 1993; Loucks & Heath, 1994). Low T_3 is a consistent finding in women with anorexia nervosa, concurrent with reductions in RMR. Conversely, weight gain in these patients increases T_3 concentrations and REE independent of RMR (Onur et al., 2005). The effects of T_3 on bone may be exerted directly via thyroid hormone receptors in bone, or through indirect regulation of the GH/ IGF-I axis (Huang et al., 2000; Lakatos et al., 2000). Thyroid hormone receptors are present on osteoblasts osteoclasts and chondrocytes (Waung et al., 2012). Mice devoid of these thyroid hormone receptors have retarded maturation of long bones and low trabecular and total BMD (Kindblom et al., 2005). Reductions in bone formation (PICP and OC) due to reduced EA occurred in parallel with reductions in T_3 levels in sedentary women

(Ihle & Loucks., 2004). Whether similar T_3 reductions following short-term low EA occur in physically active individuals in relation to changes in bone metabolism remain unknown.

2.12.5. Ghrelin

Ghrelin is a peptide secreted by the gastric fundus and has a critical role in short-term energy homeostasis and, thus, it is proposed as an appetite regulator (Kojima & Kangawa, 2005). Elevated ghrelin concentrations have been reported in exercising women under energy deficiency (De Souza et al., 2004) and anorexic individuals compared to normal-weight controls (Lawson et al., 2010). No previous study has, however, documented these changes in parallel with alteration in bone-related outcomes when energy deficiency is induced in participants with normal function (*i.e.*, bone and reproductive health). Osteoblasts express ghrelin receptors and ghrelin stimulates osteoblast differentiation and proliferation in vitro (Delhanty et al., 2006). In animal models, ghrelin increases BMD independently of food intake or weight gain (Fukushima et al., 2005). In humans, fasting ghrelin levels are negatively correlated with β-CTX (Huda et al., 2007), and treatment with an oral ghrelin mimetic has been associated with a modest increase in femoral neck BMD, but with no change in spine or total hip BMD (Nass et al., 2008).

2.12.6. PYY

PYY is a gastrointestinal peptide secreted from L-cells in the intestine in response to caloric intake. Thus, PYY signals satiety and inhibits nutrient intake (Batterham et al., 2003). PYY is elevated in patients with anorexia nervosa (Misra & Klibanski, 2011) and females with exercise-related hypothalamic amenorrhea (Scheid et al., 2011). PYY actions on bone are mediated through the Y2 receptor and Y2 receptor knockout mice have increased bone formation, bone mass and trabecular bone volume (Russell et al., 2009). These data suggest that elevated PYY may be associated with bone loss. Indeed, higher PYY levels are associated with lower levels of bone formation markers and lower lumbar BMD Z-scores in adolescent athletes (Russell et al., 2009). In adult exercising women, higher PYY levels have also been associated with lower hip and whole body BMD Z-scores (Scheid et al., 2011). Further studies are needed to investigate these relationships in interventional studies in physically active men and women.

2.12.7. GLP-1 and GLP-2

GLP-1 and GLP-2 are secreted by L-cells in the intestine, in response to feeding (Naot & Cornish, 2014). Anorexic adolescents have low GLP-1 levels (Tomasik et al., 2004), but not significantly different GLP-2 levels (Wojcik et al., 2010) compared to normal weight

controls. GLP-1 receptors are expressed in bone marrow stromal cells and pre-osteoblasts. *In vitro* studies have not shown direct effects of GLP-1 on either osteoblasts or osteoclasts (Yamada et al., 2008). However, GLP-1 stimulates calcitonin secretion and thus, indirectly inhibits bone resorption (Yamada et al., 2008). GLP-2 receptors are present on osteoclasts (Pacheco-Pantoja et al., 2011). Subcutaneously injected GLP-2 in postmenopausal women resulted in a significant dose-dependent reduction of bone resorption as assessed by β-CTX, but did not alter bone formation evaluated by OC or P1NP (Henriksen et al., 2007). Current understanding of GLP-1 and GLP-2 changes in response to energy deficiency in physically active men and women is incomplete and warrants further investigation in relation to bone-related outcomes.

2.13. Summary

Research on the Female Athlete Triad has been conducted for over 20 years and important advances have been made to our understanding of this condition during this time. These include recognition of low EA as the underlying factor for reduced bone health and reproductive function (Nattiv et al., 2007; De Souza et al., 2014a). Recent advances in our understanding have led to the introduction of the RED-S model to describe a spectrum of negative consequences arising from low EA and an expansion to the affected populations *(i.e.,* men) (Mountjoy et al., 2014; 2015). There are, however, several knowledge gaps remaining. Bone health is one of the three initially proposed Female Athlete Triad components, but there remains a lack of well-controlled systematic investigations into the effects of low EA (attained by diet, exercise or both) on bone metabolism, mass and strength in physically active women or men. An understanding of the effects of low EA on regulatory and reproductive hormones in physically active, normal-weight individuals also remains limited, with our current knowledge often being simply extrapolated from what we know for chronic conditions of energy deficiency. The available evidence for men are more limited than for women and direct comparisons of regulatory hormone responses to low EA, in parallel with the characterisation of bone responses, are lacking. It is important for future research to define the responses of the regulatory hormones to low EA and to relate these directly to responses of the bone tissue. The current work, presented in this thesis, will provide further insight into the effects of low EA on bone metabolism and health in physically active individuals.

Chapter 3. General Methods

This chapter provides a summary of the general methods used throughout all studies, with detailed descriptions of the procedures and justification for the methods and analyses used. Specific research designs, inclusion/exclusion criteria and statistical analyses are presented in the relevant experimental chapters.

3.1. Ethical approval

All experimental studies had approval from the Nottingham Trent University Ethical Review Committee (Humans). The studies described in Chapters 7 and 8 had dual approval from the Research Ethics Committee East Midlands - Derby and the Nottingham Trent University Ethical Review Committee (Humans). Approval references are detailed below:

- Repeatability of exercise energy expenditure measurements in physically active individuals by indirect calorimetry- Nottingham Trent University Ethical Review Committee (Humans), Application Number: 411
- Bone Turnover in response to short-term energy restriction in men and women- Nottingham Trent University Ethical Review Committee (Humans), Application Number: 269
- Effects of short-term energy restriction achieved by diet or exercise on bone turnover, muscle function and cognition- Research Ethics Committee reference: 14/EM/1156 Intergraded Research Application System project ID: 154899

3.2. Recruitment

Recruitment of participants occurred through posters, emails and meetings with sports clubs and coaches and by word-of-mouth.

3.3. Questionnaires

3.3.1. Health screen

A standard health screen questionnaire (Appendix 1) was used to confirm that participants were able to participate in the exercise sessions, did not suffer from any conditions known to affect bone health and were not taking medications that could influence bone metabolism and health (for a detailed description please see inclusion/exclusion criteria in each experimental chapter).

3.3.2. Reproductive function

Reproductive function, including previous menstrual irregularities, use of contraception, frequency and duration of menstrual cycles, was assessed using a standard menstrual cycle (Chapter 4 and 7) or OCP questionnaire (Chapter 8). Participants using the non-hormonal coil (copper coil) were included and identified as eumenorrheic provided they met all of the other criteria for regular menstrual cycles (please see section 3.3.2.1.). OCP users were recruited if they were taking a monophasic, low dose ethinyl-oestradiol combined OCP for at least 3 months prior to participation and were planning to continue taking the combined OCP for at least 2 months.

3.3.2.1. Menstrual function questionnaire

Menstrual cycle details were assessed using a questionnaire (Appendix 2), which included questions about menstrual frequency and length, bleeding length and previous OCP 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. 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 all studies to ensure that existing reproductive disturbances did not affect the findings.

3.3.2.2. OCP questionnaire

OCP characteristics were assessed using an OCP questionnaire (Appendix 3-Chapter 8), which included questions regarding OCP type, brand and length of use. Participants were included if they were taking a monophasic, low dose combined OCP (containing less than 50μg oestradiol and synthetic progestin) for at least 3 months prior to recruitment. Women with hormonal intrauterine devices or implants, women on non-low dose monophasic combined OCP (*i.e.*, biphasic, progesterone only OCP) (please also see section 8.2.1) were excluded from participation. The combined OCP was chosen over others, as it is the most common used OCP (Lader et al., 2009).

3.3.3. SCOFF questionnaire and weight history

The SCOFF questionnaire is a screening tool for eating disorders (Morgan et al., 1999). It consists of 5 simple questions on key features of anorexia nervosa and bulimia nervosa and the letters in the acronym are taken from key words of these questions; **S**ick, **C**ontrol, **O**ne stone, **F**at, **F**ood. It is a validated, highly sensitive, but moderately specific instrument.

Although it is not a diagnostic tool, a score greater than 2 yes responses is indicative of a potential eating disorder; thus, participants with a score greater than 2 yeses were excluded from participation (Study 1 & 2). The SCOFF questionnaire has been used previously in clinical settings and is considered to satisfactorily identify cases and exclude non-cases of common eating disorders (Tury et al., 2010). Additional typical questions used in dietetic practice were used to exclude participants with recent weight changes with potential effects on BTM (Ihle & Loucks, 2004) (Appendix 4).

3.3.4. Physical activity questionnaire

The short version of the International Physical Activity Questionnaire (IPAQ) was used to assess physical activity levels of the participants at the time of recruitment (Craig et al., 2003) (Appendix 5). The IPAQ short form asks about three specific types of activity; walking, moderate-intensity activities and vigorous intensity activities; frequency (measured in days per week) and duration (time per day). Each type of activity is weighted by its energy requirements defined in metabolic equivalents (MET- are multiples of the resting metabolic rate) to yield a score in MET-minutes·week^{-1}. A MET-minutes·week^{-1} was computed by multiplying the MET score by the minutes performed as detailed below. For each individual, the scores for each type of activity were calculated.

Walking MET-minutes·week^{-1} = 3.3 * walking minutes * walking days

Moderate MET-minutes·week^{-1} = 4.0 * moderate-intensity activity minutes * moderate days

Vigorous MET-minutes·week^{-1} = 8.0 * vigorous-intensity activity minutes * vigorous-intensity days

Three categories of total PA were determined based on the IPAQ and METs: low (<600 MET-minutes·week^{-1}); moderate (≥600 MET-minutes·week^{-1} and <3000 MET-minutes·week^{-1}); and high physical activity levels (≥ 3000 MET-minutes·week^{-1}) (IPAQ, 2005). Participants with moderate and high physical activity levels were recruited for the current programme.

3.4. Anthropometric measurements

3.4.1. Height

Height was measured to the nearest 0.1 cm (Stadiometer, Seca, Hamburg, Germany). Participants removed their footwear and stood flat footed with their heels against a back plate.

3.4.2. Body mass

Body mass was measured to the nearest 0.1 kg using electronic scales (Seca, Birmingham, UK). Participants wore minimal clothing (*i.e.*, t-shirts, shorts or leggings) and were instructed to wear the same clothing for repeated measurements within each experimental condition (pre and post). In Study 3 and 4 (Chapters 7 and 8) participants consumed 500 mL of water upon wakening in order to account for potential body mass changes due to variability in hydration status. This information was verbally confirmed with participants when they came to the laboratory. Body mass measurements were conducted in the morning following blood testing.

3.4.3. BMI

BMI was calculated using the standard formula: body mass (kg) divided by height squared (m^2). Participants were included if they had a BMI between 18.5 and 30 $kg \cdot m^{-2}$. Conversely, individuals with a BMI of <18.5 or >30 $kg \cdot m^{-2}$ were excluded from participation.

3.5. DXA

A DXA scan was performed, using a Lunar iDXA scanner (Lunar, GE Healthcare) to assess body composition and to determine baseline bone characteristics, namely total body BMD, and total body BMC.

3.5.1. Quality control assurance

A quality assurance check was performed using standard techniques at the beginning of each day to ensure that the measurements were accurate and precise.

3.5.2. Total body scan measurement

Prior to DXA measurements, participants were asked to remove their shoes and any items that could lessen the X-ray beam, including belts, earrings, zippers, snaps and buttons. They were instructed to wear a sport outfit (t-shirts, shorts or leggings) for the DXA scan. If

participants did not have the exact outfit, they were provided with some spare sport outfit by the study investigators. Several studies have demonstrated that a number of factors including hydration status, fluid fluctuation and food consumption may affect DXA measurements (Nana et al., 2015; Rodriguez-Sanchez et al., 2015). In order to minimise these factors, participants were instructed to consume 500 mL of water and have their last meal at least 3 hours before the measurement.

Participants' details (forename, surname, date of birth, participant's ID, weight, height, race and sex) were inputted and used to create a participant's profile. The operator provided instructions about right positioning on the scanner table. Participants laid within the scanning area and in the centre of the scanner table, with their head, spine and pelvis aligned to the reference centreline. Hands were positioned next to the body and within the scanning area and legs were placed straight with feet together. Straps were placed around the participant's legs (knee level and ankles) to limit movement during the measurement and to maintain positioning.

All scans were conducted and analysed by the same operator to limit inter-observer variability. For Study 1 and 2 (reported in Chapter 5-7) the DXA scans were carried out at the Clinical Skills Suite at the University of Derby, as a DXA scanner was not available at NTU at this time. For Study 3 and 4 (reported in Chapter 8 and 9) all scans took place at NTU. DXA scans have been shown to be capable of producing highly reliable estimates of total and regional body composition; CV <1% for repeated measures (Hind et al., 2011).

3.5.2.1. Body composition

The following body composition data were collected: body mass (kg), tissue (% fat), region (% fat), tissue (g), LBM (g), FFM (g) and BMC (g). LBM was used in the prescription of diet and exercise in all experimental conditions. Tissue (% fat) and FFM were used as descriptive participant characteristics of the participants.

3.5.2.2. BMD and BMC

For the purposes of this thesis, neither BMC nor BMD were considered main outcome measures. However, BMC and BMD at the onset of each experiment were provided as descriptive participant characteristics. The DXA output included BMD of total, total right and total left body, arms, legs and trunk (right and left sides), ribs, pelvis and spine. T-scores and Z-scores were also provided for total BMD (please see section 2.11.2.).

3.6. Aerobic fitness test

Participants performed a sub-maximal incremental test on a motorised treadmill (HP Cosmos, Germany), consisting of a speed lactate test and a VO_2 peak test previously developed by Jones and Doust (1996) to establish the relationship between running speed and oxygen consumption during level running. During the test, participants wore a standard heart rate monitoring device (Polar FT-2, Finland) using a strap fitted around their chest. This provided continuous recordings of heart rate during exercise. Furthermore, participants were fitted with a facemask to ensure normal breathing throughout the exercise. Expired gas samples were continuously collected and analysed by a breath-to-breath automated gas analysis system (ZAN, nSpire Health); for more details about calibration of the system and analysis please see section 3.8.2.and 3.8.3.

Briefly, participants first completed a speed lactate incremental test, which was continuous in nature and consisted of graded exercise steps of 3 minutes. Participants commenced running at a 0 gradient at an easy-pace speed, between 7 and 9 $km \cdot h^{-1}$ depending on their training background, and thereafter intensity increased by 1 $km \cdot h^{-1}$ every 3 minutes. Participants had capillary blood samples (taken by finger prick) taken at the end of each incremental stage to determine blood lactate levels (Yellowstone Scientific Instruments, Big Sky, Montana). The test terminated when changes $\geq$ 1 $mmol \cdot L^{-1}$ in blood lactate concentration were detected between stages.

After the incremental exercise test, participants were given 10 minutes to recover. Following this, participants performed a ramp test to determine maximum oxygen uptake. The ramp test was continuous in nature and consisted of 1-minute stages. The initial speed was determined according to the speed reached during the incremental exercise test and the grade increased by 1% every minute, from a starting gradient of 0%, until volitional exhaustion was achieved (Jones & Doust, 1996). At the end of this test, participants had a rating of perceived exertion of 9 or 10 out of 10. Additionally, the majority, but not all, of the participants achieved a respiratory exchange ratio greater than 1.10 (Howley et al., 195; Midgley et al., 2009).

The running speed at each stage of the speed lactate incremental test was plotted against oxygen consumption ($mL \cdot kg \cdot^{-1} min^{-1}$) to determine the sub-maximal relationship between speed and oxygen consumption. The computation of this regression line in combination with VO_2 peak results were used to estimate the running speed corresponding to 70% VO_2 peak at

0% gradient during the main experimental, exercise protocol based on the regression line of VO_2 and speed.

3.7. Habitual dietary intake

3.7.1. Three-day food records

Before experimental trials, participants were asked to weigh and record the amount of all food and beverages consumed over a 3-day period to provide information about their habitual DEI. Three-day food records have been used in EA studies (Ihle and Loucks, 2004; Reed et al., 2013) and have been shown to adequately capture habitual macronutrient intake (Magkos & Yannakoulia, 2003).

All participants were shown by a registered dietitian how to complete the dietary record to the level of detail required to adequately describe the food and amounts consumed, including the name of the food (brand name), preparation methods, recipes for food mixtures and complicated dishes, and portion sizes. Written instructions and examples were also provided (Appendix 6). Participants were provided with food record forms consisting of 4 columns: food/drink item, amount eaten (g), details, amount of leftovers (g) (Appendix 7).

Several strategies were implemented to aid the collection of the dietary data:

1. Participants were instructed to maintain their normal food intake and eating patterns during the recording period.
2. The importance of recording at the time of eating was highlighted. This limits the problem of omission and provides a more detailed description of the food.
3. To minimise the errors in estimating food quantities, participants were asked to weigh their food using electronic scales (Aquatronic Kitchen Scale, Salter, UK-Study 1 & 2; HOME Electronic Kitchen Scale; Argos, UK- Study 3 &4). Participants were shown how to use the scales correctly and how to record the weight of food items included in more complex dishes. Participants were encouraged to carry the scales provided by the investigators even when eating out. Alternatively, instructions were provided for estimating quantities when eating out, when weighing food was not possible. Some foods have pre-defined units (*i.e.*, one slice of bread), which was helpful for participants when recording the quantity. Household measures (tablespoons, teaspoons and cups) were also recommended, but strictly only in cases where participants did not have access to the scales provided.

4. Often, participants do not provide enough information about specific food items or food groups (*e.g.*, sauces, type and amount of fat used during food preparations, beverages and complementary food items such as sugar and milk in tea or coffee). In an effort to collect this detailed information, participants received specific verbal and written instructions on how to record these food items (Appendix 6).
5. Participants were encouraged to keep nutritional labels, provide restaurant information when eating out and record recipes when describing their food consumption.
6. At the end of the recording period, food records were reviewed and, when needed, the participants were asked to clarify entries and crosschecked for forgotten food/meals (Study 1 and 2).

3.7.2. Dietary analysis

All dietary analyses were performed by a registered dietitian using Microdiet^{TM}. DEI (kcal·d^{-1}), absolute macronutrient intake (carbohydrates, proteins, fat; g·d^{-1}) and percent contribution of macronutrients to daily DEI were determined for each participant.

3.8. Measurement and estimations of EE

3.8.1. Measurement of lifestyle EE-Accelerometers

Lifestyle physical activity was measured using an Actigraph accelerometer (WGT3X-BT; Actigraph, East Chase Street. Pensacola, FL). The Actigraph accelerometer has been widely used in previous short-term laboratory studies (Koehler et al., 2016; Hagobian et al., 2009). Previous studies have shown that 3 days of physical activity records using accelerometers achieve 80% reliability for the measurement of physical activity (Matthews et al., 2002). Participants were instructed to wear the accelerometers for all waking hours, except when bathing. They were also directed to attach the accelerometer on the right hip, to ensure consistency and limit variability.

Participants' characteristics (code, weight, height, date of birth, race) were entered manually into the system software. The Freedson combination kcal equation was chosen to translate the accelerometer output into EE (Freedson *et al*., 1998). This equation has been previously shown to provide close estimates of energy expenditure during both light and moderate physical activity compared to other equations that significantly under- or over-estimate the time spent in activities of these intensities (Crouter et al., 2006). All equations appear to underestimate time spent in vigorous exercise (Crouter et al., 2006), however, the accelerometers in this thesis were used to monitor lifestyle physical activity, which is

expected to vary from light to moderate intensity, rather than systematic exercise (high intensity), which was performed in the laboratory and measured using an indirect calorimetry system.

3.8.2. Measuring EEE

EEE was measured using a breath-to-breath automated gas analysis system (ZAN, nSpire Health). ZAN is a ventilated open-circuit system and has components to collect expired air, measure flow rate, analyse gas concentrations and pump air through the system (Levine et al., 2005). Prior to each measurement, the gas analysis system was calibrated with oxygen and carbon dioxide as a mixture of known concentrations and the volume transducer was calibrated with a 3-L calibration syringe according to the manufacturer's guidelines.

The exercise experimental protocol involved repeated bouts of running for approximately 60 minutes (Study 1 & 2) and 2 x 60 minutes (approximately) (Study 3 & 4) on consecutive days. Each bout was conducted as 3-to-5, 15-minutes sessions, separated by a 5-minute rest. Exercise intensity was controlled by setting the treadmill speed and/or grade to reach 70% VO_2 peak for each participant (please see section 3.6). Small adjustments were made to maintain the intensity at 70% VO_2 peak. During the exercise protocol, the participants wore a facemask and told to breathe normally throughout the exercise. Expired gas samples were continuously collected, averaged over 20 seconds and analysed following test completion. The required duration of exercise was determined using the oxygen uptake values and respiratory exchange ratio. Data previously collected in our laboratory (please see section 3.8.3) using the same techniques, have shown that EEE of the same duration and intensity is similar when repeated under the same conditions for the same individual. Therefore, participants did not wear the facemask during exercise on the following days of the experimental protocol.

3.8.3. Day to day variability in EEE in physically active individuals by indirect calorimetry

3.8.3.1. Introduction

Due to the prolonged and repeated exercise bouts, we anticipated that some participants may not feel comfortable when wearing a facemask for each exercise session during the main experimental protocol (Study 1-4). If the exercise protocol employed is able to produce consistent measures of EEE, following an initial EEE measurement participants would be able to perform exercise bouts without wearing a face mask. The reliability of a protocol

reflects the consistency of the data when the measurements are collected on a number of occasions under identical conditions (Atkinson & Nevill, 1998). Several factors such as circadian rhythm, consistency of the equipment used and participant and researcher error may introduce measurement errors. Atkinson and Nevill (1998) have recommended that reliability is the amount of measurement error considered satisfactory for the use of an analysis system in practice. The aim of this study was to compare EEE values measured using the same system of indirect calorimetry as in the main experimental studies, on two occasions under the same conditions.

3.8.3.2. Methods

Thirty participants (Table 3.1.) volunteered to participate in this experiment having provided informed consent (Appendix 8-Paticipant's Information Sheet, Appendix 9-Informed Consent Form). Participants for this study were representative of the population used for the main studies of this PhD programme. Participants were included if they were Caucasian, aged 18-40 years, non-smokers, had a BMI between 18.5 and 30 $kg \cdot m^{-2}$, had not sustained a bone fracture within the previous year and were currently injury free. Female participants were included if they were eumenorrheic or were taking a monophasic, low dose ethinyl-oestradiol combined OCP. For female participants, exclusion criteria were extended to include: breastfeeding, pregnancy, any type of other hormonal contraception and self-reported short (<24 days), long (>35 days) or irregular menstrual cycles (please see section 3.3.2.1 and 3.3.2.2.- Appendix 2 and 3).

3.8.3.2.1. Research design

Participants attended a preliminary session (P) and 2-main experimental trials on two consecutive days (Visit 1 and Visit 2). The preliminary assessments were performed to establish inclusion criteria, take anthropometric measurements (please see section 3.4.1., 3.4.2. and 3.4.3.) and determine the fitness level of the volunteers (please see section 3.6.).

During Visits 1 and 2, participants completed a standardised protocol for data collection consisting of 5 min of rest, followed by 2 bouts of 15 min running at 70% VO_2 peak separated by a 5 min break on a flat treadmill. EEE during the exercise was measured using an indirect calorimetry system. The experimental conditions were standardised during Visit 1 and 2. The time of testing was controlled within participant, but not between participants. All participants were instructed to commit to testing at least 3 hours after their last meal. Food intake was recorded for Visit 1 and participants replicated it on Visit 2, in order to limit the variations in EEE due to differences in food intake. For female participants,

eumenorrheic women were tested between Day 2 and Day 7 of their menstrual cycle (Day 1: first day of bleeding) and women on combined OCP were tested between Day 2 and 7 of their combined OCP cycle (Day 1: first day of their new pack).

3.8.3.2.2. Experimental procedures

3.8.3.2.2.1. Preliminary assessment

Participants provided written and verbal informed consent (Appendix 8). They also completed a medical history questionnaire (please see section 3.3.1.- Appendix 1) and the short version of IPAQ (please see section 3.3.5.-Appendix 5). Female participants also provided information about their menstrual cycles or combined OCP cycle (Apendix 3 and 4). Upon completion of these questionnaires, anthropometric measurements were performed (please see section 3.4.1.-3.4.2.) followed by an incremental exercise test on a treadmill (please see section 3.6.) to determine their VO_{2max}.

3.8.3.2.2.2. EEE

Detailed description has been provided in 3.8.2.

3.8.3.2.2.3. DEI

The participants weighed and recorded dietary intake on Visit 1. All participants were shown how to complete the dietary record to the level of detail required to adequately describe the food and amounts consumed as described in 3.7.1. Participants were asked to replicate their dietary intake (quality and quantity) for Visit 2.

3.8.3.2.3. Statistical analysis

All data are presented as mean (1SD). All data were checked for normality according to the Shapiro-Wilk test and for homoscedasticity using Pearson's correlation coefficients (Nevill & Atkinson, 1998). EEE data were analysed using paired t-tests, intra-class correlations (ICC, 2-way fixed, repeated measures, absolute model), ratio limits of agreement (RLoA; Bland & Altman, 1986) and CV. The level of significance was set at $P<0.05$. Statistical analysis was performed using the SPSS 23.0 (Statsoft, USA).

3.8.3.3. Results

Baseline characteristics of the participants are shown in Table 3.1

Table 3.1. Baseline characteristics

Age (y)	30 (5)
Sex (female, male, %)	53, 47
Height (m)	1.74 (0.09)
Body mass (kg)	68.9 (9.1)
BMI (kg ·m^{-2})	22.8 (2.3)
VO_2 peak (ml·kg^{-1}·min^{-1})	50.3 (7.5)

Values are presented as mean (1SD).
VO2 peak: Peak oxygen uptake.

Table 3.2. Absolute and relative reliability measures of the EEE (kcal) data.

	EEE
Visit 1	315 (81)
Visit 2	311 (77)
Visit 1 (ln)	5.71 (0.28)
Visit 1 (ln)	5.72 (0.28)
Ratio LoA	1.145
CV (%)	3.14
ICC (CI)	0.983 (0.964-0.992)
t-test	0.40

Values are presented as mean (1SD); LoA: Limits of Agreement, CV: Coefficient Variation, ICC: Interclass Correlation; CI: Confidence Interval.

There was no significant difference between Visit 1 and 2 for EEE (315 ± 81 and 311 ± 77 kcal; P =0.40). ICC between trials was r = 0.983 (0.964 to 0.992) for EEE. The CV (%) was 3.14% for EEE measurements. For a typical EEA measurement of 313 kcal, there is 95% likelihood that a second measurement would have a value between 284 and 342 kcal.

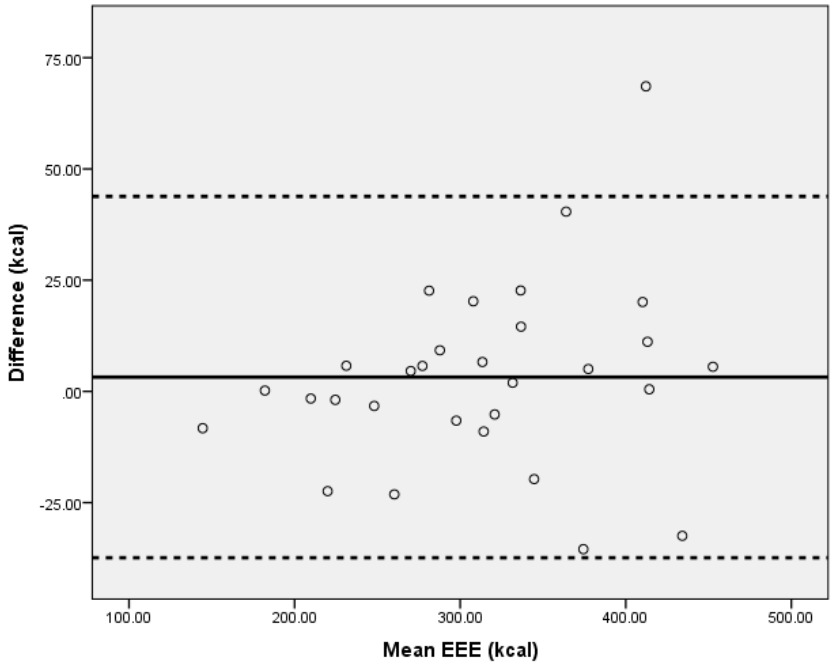


Figure. 3.1. A Bland-Altman plot for EEE (kcal) for Visit 1 and 2. Systematic bias indicated by dotted line and 95% limits of agreement indicated by dotted lines. EEE: Exercise Energy expenditure

3.8.3.4. Discussion

EEE measurements were not significantly different between visits (P = 0.40). The consistency of the EEE measurements was demonstrated by narrow limits of agreement, excellent ICC of 0.983 and a CV of 3.14. The current data provide evidence that EEE measurements using a breath-by-breath system are reliable in a healthy, physically active population representative of the population used in the main experimental studies. Therefore, we suggest that this protocol can be used to produce comprehensive and consistent data and that the equipment used in this study provides a satisfactory measurement of EEE. As such, following an initial measurement of EEE, exercise sessions were completed without the facemask and the use of the breath-by-breath analysis system (Chapter 4-8).

3.9. EA

EA was defined as DEI minus EEE. EA was normalised to kilograms of LBM to consider metabolically active tissue and account for individual differences in body composition. Healthy, regularly menstruating, sedentary women achieve energy balance at an EA of 45 $kcal \cdot kgLBM^{-1} \cdot min^{-1}$ (Loucks & Thuma, 2003; Ihle & Loucks, 2004). This level was used as the "controlled balanced" level in all studies (Study 1-4) in order to allow comparisons with previous literature (Loucks & Thuma, 2003; Ihle & Loucks, 2004).

3.10. Variables of EE

TEE was calculated as the sum of RMR, diet-induced thermogenesis, lifestyle EE and EEE. RMR was estimated using Harris-Benedict revised equations, which consider age, weight, height and sex (Rosa & Shizgal, 1984). These are:

Men: RMR ($kcal \cdot d^{-1}$) = 88.362 + (13.397 x weight in kg) + (4.799 x height in cm) - (5.677 x age in years)

Women: RMR ($kcal \cdot d^{-1}$) = 447.593 + (9.247 x weight in kg) + (3.098 x height in cm) - (4.330 x age in years)

Diet-induced thermogenesis can be defined as the energy required for nutrient digestion, absorption and storage and was calculated as 10% of DEI, according to estimations in healthy individuals following a mixed diet (Westerterp et al, 2004).

3.11. Primary and secondary outcomes

Markers of bone turnover were determined as primary outcomes of the study. β-CTX was chosen as a marker of bone resorption and P1NP as marker of bone formation. These markers have been proposed as reference standards for bone resorption and formation respectively by the International Osteoporosis Foundation and the International Federation of Clinical Chemistry and Laboratory Medicine (Vasikaran et al., 2011) and National Bone Health Alliance (US, Bauer et al., 2012) (please see section 2.5.4.5). Both markers have been shown to be specific for the procedure they represent and are widely used in clinical (Vasikaran et al., 2011) and research settings (Banfi et al., 2010). The ratio between P1NP and β-CTX was calculated in order to provide a numerical quantification of bone turnover (BT ratio). Similar ratios have been calculated in the existing literature (Vincent et al, 2002; Lombard et al, 2012b). Markers of calcium metabolism, energy regulatory hormones and reproductive hormones (Table 3.4.) were determined as secondary outcomes- rationale for these measurements has been provided in sections 2.6.1., 2.6.3. and 2.12.

Table 3.4. Markers of calcium metabolism, energy regulatory and reproductive hormones

Marker	Rationale
Markers of calcium metabolism	
PTH	Principal hormone regulating calcium homeostasis and bone remodelling.
Mg	Key micronutrient for bone homeostasis. Required for PTH interpretation
ACa	Key micronutrient for bone homeostasis. Required for PTH interpretation
PO_4	Key micronutrient for bone homeostasis Required for PTH interpretation.
Regulatory and reproductive hormones	
Insulin	A peptide hormone, produced by beta cells of the pancreas; offers potential link between skeletal homeostasis and energy regulation. Low insulin levels are associated with decreased bone formation.
T_3	A thyroid hormone that decreases in low EA. Low T_3 levels are associated with decreased bone formation.
IGF-1	A growth-promoting polypeptide with essential role in bone homeostasis. Changes in IGF-I levels may contribute to the disturbance of bone turnover under low EA. Low IGF-1 levels are associated with decreased bone formation.
Leptin	A peptide, product of ob gene; reflects energy deficiency adaptations. Decreased levels are documented in models of chronic undernutrition. It offers potential link between skeletal homeostasis and energy regulation.
GLP-2	A peptide released from the intestinal mucosa in response to food intake
Sclerostin	A measure of bone formation. Protein produced by osteocytes. It plays a key role in Wnt pathway, which regulates bone formation.
Oestrogens	Major sex steroid in women. Decreased levels and bioavailability of oestrogens under periods of energy restriction. Low levels of oestrogen are associated with decreased bone resorption in women.

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.

3.12. AUC

Area under the curve (AUC) with respect to baseline (BASE) was calculated for all biochemical markers from the percentage change data (Zweig and Campbell, 1993).

3.13. Blood sampling and storage

Blood samples were obtained at the same time of day for each participant (please see details in each experimental chapter) after an overnight fast (from 20:00 pm in the previous evening). Blood was drawn from a vein in the forearm by a trained phlebotomist using

standardised procedures. Plasma samples were centrifuged immediately at 3000 revolutions per minute for 10 min at 4ºC. Venous blood was also dispensed into serum tubes and allowed to clot at room temperature for 30 minutes before being centrifuged under the same conditions. Resultant plasma and serum were aliquoted into Eppendorf tubes and stored at -80ºC. *β*-CTX, PINP, PTH and IGF-1 were analysed in plasma. Serum was analysed for sclerostin (Study 1 & 2), leptin, insulin, T_3, GLP-2 (Study 1 & 2), 17*β*-oestradiol, albumin, Ca, PO_4 and Mg.

3.14. Biochemical analysis

β-CTX, P1NP, PTH, T_3 and 1*7β*-oestradiol were measured using ECLIA (Roche Diagnostics, Burgess Hill, UK) on a Cobas e601. Inter-assay CV for *β*-CTX was <3% between 0.2 and 1.5 $\mu g \cdot L^{-1}$, with sensitivity of 0.01 $\mu g \cdot L^{-1}$. P1NP inter-assay CV was <3% between 20-600 $\mu g \cdot L^{-1}$ and sensitivity of 8 $\mu g \cdot L^{-1}$. PTH inter-assay CV of <4% between 1-30 $pmol \cdot L^{-1}$ and sensitivity of 0.8 $pmol \cdot L^{-1}$. Sclerostin was measured using an ELISA supplied by Biomedica GmbH (Vienna Austria) with a sensitivity of 2.6 $pmol \cdot L^{-1}$ established from precision profiles (22% CV of duplicates) and a CV of <15% across the range 25-95.0 $pmol \cdot L^{-1}$. T_3 inter-essay CV of <1% between 2.0-3.1 $nmol \cdot L^{-1}$ and detection limit of 0.3 $nmol \cdot L^{-1}$. The inter-assay CV for 1*7β*-oestradiol was <3% between 214.3-2156.7 $pmol \cdot L^{-1}$ and detection limit of 18.4 $pmol \cdot L^{-1}$. Leptin was measured using ELISA (Biovendor, Czech Republic) and had an inter-assay CV of <7% across the range 1-50 $\mu g \cdot L^{-1}$ and sensitivity 0.2 $\mu g \cdot L^{-1}$. 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 $ng \cdot mL^{-1}$ and sensitivity of 4.4 $ng \cdot mL^{-1}$. 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 $ng \cdot mL^{-1}$ and a detection limit of 0.5 $ng \cdot mL^{-1}$. Insulin was measured using ECLIA on a Cobas e601 (Roche Diagnostics, Burgess Hill, UK), inter-assay CV is <6.1% across the range 44-505 $pmol \cdot L^{-1}$ and sensitivity is 1.8 $pmol \cdot L^{-1}$. 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 $mmol \cdot L^{-1}$ for Ca, 10-70 $g \cdot L^{-1}$ for albumin and 0.10-6.46 $mmol \cdot L^{-1}$ for phosphate. Mg was measured using a commercial assays supplied by Roche Diagnostics and analysed on a COBAS c501. The inter-assay CV was 0.9% across the range 0.1-2.0 $mmol \cdot L^{-1}$ and the sensitivity was 0.05 $mmol \cdot L^{-1}$.

Chapter 4. Bone metabolic response to short-term low energy availability in physically active women

4.1. Introduction

Physically active individuals commonly follow dietary energy restriction during training, which can result in low EA. These practices put them at risk for sustaining a stress fracture injury (Bennell et al., 1996a; Warden et al., 2014) and their bone health is a topic of concern both during and after their careers (Nattiv et al., 2007; Mountjoy et al., 2014). Interrelationships between low EA, reproductive function and BMD in women have been described under the Female Athlete Triad (Nattiv, 2007; De Souza et al., 2014a). A number of female athletes partaking in weight sensitive sports follow low EA and present with low BMD (Nattiv et al., 2007; Loucks et al., 2007; 2011; Melin et al., 2015). High prevalence rates of stress fracture injuries have been reported in female athletes (Wentz et al., 2011), but also female military recruits (Wentz et al., 2011; Beck et al., 2000), who are similarly exposed to periods of prolonged exercise without adequate DEI (Tharion et al., 2005; Hoyt & Friedl, 2006). Factors associated with energy deficiency including low EA, low body weight/BMI, disordered eating and rapid weight loss are emerging as risk factors for sustaining a stress fracture injury (Barrack et al., 2014; Warden et al., 2014) and an osteoporotic fracture in later life (Compston et al., 2016). As such, understanding the effects of low EA on bone metabolism and health is important for implementing prevention and treatment strategies for bone related disorders in these physically active women.

Low BMD is likely to reflect uncoupled bone turnover (decreased bone formation, increased bone resorption or a combination of the two) following low EA (Zanker & Swaine; 1998a; Villareal et al., 2016). Initial bone turnover responses to low EA in physically active women and their potential long-term effects are unknown. Short-term energy restriction has unfavourable effects on bone, with detrimental effects of short-term fasting on bone turnover having been shown (Grinspoon et al., 1995). Decreased bone formation, at different levels of low EA (30, 20 and 10 kcal·kgLBM^{-1}·d^{-1}), and increased bone resorption, with severely restricted EA (10 kcal· kg LBM^{-1}·d^{-1}) over 5 days, has been shown in sedentary women when compared to balanced EA (45 kcal· kg LBM^{-1}·d^{-1}) (Ilhe & Loucks, 2004). Some amenorrheic athletes report EA at ~16 kcal·kgLBM^{-1}·d^{-1} (Thong et al. 2000), making it important to explore the effects of this level of reduced EA on bone turnover in physically active women.

Physiological adaptations to low EA involve alterations in energy regulatory hormones; decreases in T_3, IGF-1 and leptin; as well as elevations in ghrelin and PYY (Ihle & Loucks, 2004; Zanker and Swaine, 2000; De Souza et al., 2008; Sheid et al., 2011). Low EA results in suppression of ovulatory cycles, inhibition of GnRH, reductions in LH pulsatility and

oestrogen levels in women (De Souza et al., 2004; Loucks & Thuma, 2003). Changes in energy regulatory hormones and hormones indicative of reproductive function have been suggested to affect bone metabolism and BMD by acting independently or synergistically in women with energy and/or oestrogen deficiency (De Souza et al., 2008; Ihle & Loucks, 2004). Their individual contribution and complex interplay in mediating bone responses, however, remain to be elucidated.

To address these issues, we conducted a randomised, crossover study to investigate the effects of short-term, low EA at 15 $kcal \cdot kgLBM^{-1} \cdot d^{-1}$, achieved by combined dietary energy restriction and exercise, on BTMs in physically active, eumenorreic women. A secondary goal was to investigate changes in regulatory and reproductive hormones and markers of calcium metabolism that may mediate potential differences in BTMs. It was hypothesised that bone formation would be decreased and bone resorption would be increased by low EA.

4.2. Methods

4.2.1. Participants

Eleven eumenorrheic women (Table 4.2.) volunteered to participate in this experiment having provided informed consent (Appendix 10-Paticipant's Information Sheet, Appendix 11-Informed Consent Form). Participants were included if they were Caucasian, aged 18-35 years, non-smokers, had a 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 in writing via a health screen (please see section 3.3.1.- Appendix 1) and SCOFF questionnaire (Morgan et al., 1999) (please see section 3.3.3.- Appendix 4.). Exclusion criteria included breastfeeding, pregnancy, any type of hormonal contraception and self-reported short (<24 days), long (>35 days) or irregular menstrual cycles (please see section 3.3.2.). Participants regularly performed ≥3 hours of moderate to vigorous physical activity per week and had moderate and high physical activity levels as determined by the IPAQ (please see section 3.3.4.).

4.2.2. Experimental design

This study was performed using a randomised (Standard Latin squares for 2 x 2), counterbalanced, crossover, design. Participants completed a 9-day experimental period (D1-D9) on two occasions (condition 1 and condition 2). Participants followed a controlled balanced (CON: 45 $kcal \cdot kg^{-1}LBM \cdot d^{-1}$) and a restricted EA (RES: 15 $kcal \cdot kg^{-1}LBM \cdot d^{-1}$). Both EAs were achieved by manipulating diet and exercise. Participants completed exercise sessions at 70% VO_2 peak resulting in EEE of 15 $kcal \cdot kg^{-1}LBM \cdot d^{-1}$. DEI provided 60 and 30 $kcal \cdot kg^{-1}LBM \cdot d^{-1}$ in CON and RES.

During the lead-in period (D1-D3), participants recorded habitual dietary intake and lifestyle physical activity, having refrained from systematic exercise. Over the following 5 days of the protocol (D4-D8) participants undertook either CON or RES (Figure 4.1.). Participants began each condition in the early follicular phase (~28 d apart), as confirmed by 17β-oestradiol measurements on D5 (Stricker et al., 2006). Between the two conditions, participants were instructed to follow their habitual dietary intake and exercise routine. After the ~28-day gap, participants completed the second part of the study (*i.e.,* CON→ RES or RES→CON).

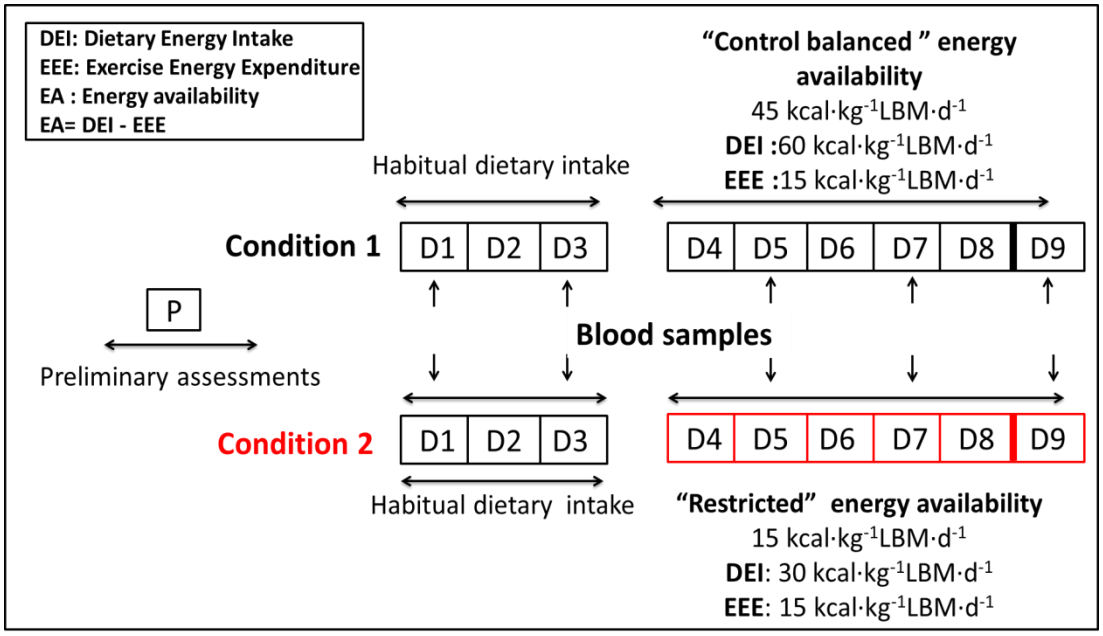


Figure 4.1. Overview of the study design. P: Preliminary assessments; D1-D8: Experimental Days. Shaded boxes denote laboratory visits and change into either the controlled (CON) or restricted (RES) period (D3-D4); adjoined boxes denote consecutive days; the thick black/red line denotes when CON/RES energy availability (EA) conditions are stopped. Arrows denote blood sampling schedule.

4.2.3. Experimental procedures

4.2.3.1. Preliminary assessments

Preliminary measurements were performed to establish inclusion criteria, take baseline measurements and determine the fitness level of the volunteers. During their first visit, participants completed a medical history questionnaire (please see section 3.3.1.-Appendix 1), SCOFF questionnaire and weight history questionnaire (please see section 3.3.3.-Appendix 4) and IPAQ (please see section 3.3.4.-Appendix 5). Following the completion of these questionnaires, participants were weighed in light clothes without shoes on a weighing scale (Seca, UK), height was obtained barefoot using a stadiometer (SECA, UK) and BMI was calculated (weight (kg) divided by the height squared (m^2)) (please see section 3.4.).

A whole body DXA axial scan was performed to assess body fat (kg, %) and LBM (kg), and determine BASE BMD ($g \cdot m^{-2}$). All scans were performed and analysed by the same operator at the Clinical Suite, Derby University in order to minimise the variability between examiners. Positioning of the participants during the DXA scan was performed according to manufacturer's guidelines, as detailed in section 3.5. Participants also performed an incremental exercise test on a treadmill (HP Cosmos, Germany) to determine their VO_2

peak, for a detailed description please see section 3.6. Accelerometers and food weighing scales were provided along with verbal and written instructions on how to complete the weighed food intake (please see section 3.7.1. and 3.8.1).

4.2.3.2. Experimental period

4.2.3.2.1. Lifestyle EE

Participants wore an accelerometer (GT3X/GT3XE, Actigraph) during all waking hours on D1-D8, except while bathing, to estimate lifestyle EE. Outside the prescribed exercise, participants were instructed to perform only light activities, such as reading or working on a computer.

4.2.3.2.2. Habitual DEI

Participants weighed and recorded food intake during D1-D3 to provide information about their habitual dietary intake. All participants were shown how to complete the food records. Written instructions and examples were also provided (please see section 3.7.1.) (Appendix 6). Dietary analysis was performed by a registered dietitian using Microdiet™ software (please see section 3.7.2.).

4.2.3.2.3. Experimental Diets

During D4-D8, 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 $kcal \cdot kg^{-1}LBM \cdot d^{-1}$ and maintained the dietary composition of each participant's habitual diet. Percentages of macronutrient intake (carbohydrates, proteins and fat) were matched with the participants' habitual DEI prior to the condition that each participant was first assigned. Three menus were designed according to the 3 records of habitual DEI and administered in a 3-day cyclic order with menu A on D4 and D7, menu B on D5 and D8 and menu C on D6. Menus included five meals in both CON and RES trials to limit the effects of food partition on bone turnover (Li & Muhlbauer, 1999). 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 or evening snack (15:00 or 17:00 h) depending on the time of exercise and dinner (21:00 h). The characteristics of the experimental diets in CON and RES trials are presented in Table 4.2. Participants provided their own food, but were instructed on the amounts, preparation and timing of the meals. They were also allowed to drink tea and coffee (without any sugar or milk) over the main experimental period and water consumption was permitted *ad libitum*. The diet plans

provided to each participant contained four columns; food- list, quantity in grams, checklist and other quantity in grams. A multivitamin, multi-mineral supplement (A - Z Tablets, Boots, Nottingham, UK) was provided during the restricted experimental period in order to provide adequate micronutrient intake and isolate the effects of energy/macronutrient restriction.

Table 4.1. Energy and macronutrient composition of prescribed experimental diets.

	CON	**RES**
DEI (kcal· d^{-1})	2490.9 (292.0)	1264.8 (150.1)
DEI (kcal·kg^{-1} LBM·d^{-1})	60.0 (0.4)	30.3 (0.2)
Carbohydrate (%)	49 (7)	49 (7)
Protein (%)	17 (5)	18 (5)
Fat (%)	34 (6)	33 (6)

Values are expressed as means (1SD).
DEI: Dietary Energy Intake; CON: Controlled; RES: Restricted.

4.2.3.2.4. EEE

During D4-D8, exercise was undertaken in order to contribute to EEE of 15 kcal·kg^{-1}LBM·d^{-1}. Participants ran on a flat treadmill 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) during the test on D4. The duration of subsequent exercise sessions (D5-D8) were calculated using the oxygen uptake and respiratory exchange ratio collected during this session, as described in section 3.8.2.

The methodological study presented in section 3.8.3. has shown that EEE during exercise of the same duration and intensity is similar when repeated under the same conditions for the same individual. During the exercise sessions on D5-D8, gas analysis was not performed, given that participants were exercising under identical conditions to the first exercise day (D4). During the exercise protocol, participants also wore a heart rate monitoring device fitted around their chest, with heart rate recorded continuously throughout each run.

4.2.3.2.5 Compliance

Compliance with the experimental diets was confirmed verbally and/or by using a self-recorded checklist. Participants reported any deviation from their prescribed meals plans.

4.2.4. Storage and analyses of blood samples

Blood samples were obtained at the same time of day for each participant between 08:15-09:00 h (maximum ± 15 min for the same participant) after an overnight fast (from 20:00 h the previous evening) on D1, D3, D5, D7 and D9. The mean value of D1 and D3 were used as BASE. Blood sample collection, processing and analysis have been previously described in section 3.1.3. β-CTX, PINP, PTH and IGF-1 were analysed in plasma. Serum was analysed for leptin, insulin, T_3, GLP-2, 17β-oestradiol, ACa, PO_4 and Mg.

4.2.5. Biochemical analysis

A detailed description of biochemical analysis is provided in section 3.14.

4.2.6. Statistical analysis

Based on results reported by Zanker and Swaine (2000), the study was sized to detect a significant change in P1NP (pre: 76.1 ± 5.8; post: 64.7± 6.0 $mg \cdot L^{-1}$, $P<0.05$) due to low EA. A *priori* power calculations showed that a minimum of 7 women were required to achieve 95% power at $P<0.05$. All data were checked for normality according to the Shapiro-Wilk test, with data being log-transformed, when not normally distributed. Baseline biochemistry markers prior to each experimental condition were compared with paired t-tests or the Wilcoxon signed-rank test. A two-way ANOVA with repeated measures was used to determine main (condition, time) and interaction (condition x time) effects for raw data of BTMs, markers of bone metabolism, regulatory and reproductive hormones in response to CON and RES EA. Tukey's tests were used as *post hoc* analyses when a significant interaction effect was shown. AUC with respect to BASE were calculated for all biochemical markers from using the percentage data from BASE (% BASE) (Zweig & Campbell, 1993). A paired t-test for normally distributed data or the Wilcoxon signed-rank test for non-normally distributed data were used to detect differences between CON and RES. Data are presented as mean (1SD) 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, the individual responses of the BTMs to RES were also examined. In order to be considered a responder, β-CTX concentrations at D9 in RES were > BASE (100%), > β-CTX concentrations at D9 in CON together with a difference >3% to account for CV of β-CTX assay. For P1NP, responders were identified if P1NP concentrations at D9 in RES were <BASE (100%), <P1NP levels at D9 in CON together with a difference >3% to account for CV for P1NP assay.

4.3. Results

4.3.1. Participant characteristics

Participant characteristics are shown in Table 4.2.

Table 4.2. Participant characteristics (n=11)

Demographics	
Age (y)	26 (5)
Height (m)	1.66 (0.05)
Body mass (kg)	59.7 (6.7)
BMI (kg ·m^{-2})	21.5 (1.5)
Body composition	
Body fat (%)	27.1 (6.2)
LBM (kg)	41.5 (4.9)
FFM (kg)	44.0 (5.1)
BMD (g·cm^{-2})	1.14 (0.11)
Training characteristics	
VO_2 peak (ml·kg^{-1}·min^{-1})	47.9 (5.5)
VO_2 peak (ml·kg LBM^{-1}·min^{-1})	69.3 (5.9)
Physical activity (MET-min·wk^{-1})	3928 (1651)
Dietary and EE characteristics	
Habitual DEI (kcal·d^{-1})[1]	2143 (361)
Lifestyle EE (kcal·d^{-1})[1]	402 (227)
24-hour EE(kcal· d^{-1})[1]	1943 (302)

Values are expressed as means (1SD).
[1] Mean values of D1-D3 prior to both experimental conditions.
BMI: Body mass index; LBM: Lean body mass; FFM: Fat free mass BMD: Bone mineral density: VO_{2max}: Maximum oxygen uptake; DEI: Dietary energy intake; EE: Energy expenditure; RMR: Resting metabolic rate; MET: Metabolic equivalents.

4.3.2. Baseline Biochemistry

Baseline *β*-CTX in RES was significantly lower than BASE levels in CON (P=0.012); with no significant differences in any other biochemical marker (Table 4.3.). Baseline BTMs concentrations were within, but at the higher end of the reference range (*β*-CTX: 0.15-0.80 µg·L^{-1}; P1NP: 25-90 µg·L^{-1}, Jenkins et al., 2013), especially those of P1NP (Table 4.3.).

Table 4.3. Markers of energy status and BASE concentrations of BTMs, markers of calcium metabolism, regulatory and reproductive hormones prior to CON and RES trials (n=11). Mean values of D1 and D3 were used as BASE prior to each experimental condition.

	CON	RES	P-value
Markers of energy status			
Body mass (kg) [1]	59.9 (6.5)	60.2 (6.2)	0.30
Habitual DEI (kcal·d^{-1})[2]	2082 (308)	2072 (349)	0.91
Lifestyle EE (kcal·d^{-1})[2]	427.6 (308.9)	375.6 (203.1)	0.52
BTMs			
***β*-CTX (µg·L^{-1})**	0.49 (0.14)	0.42 (0.11)	0.012*
P1NP (µg·L^{-1})	71.1 (15.0)	70.6 (15.1)	0.87
BT ratio	1.54 (0.44)	1.72 (0.42)	0.06
Markers of calcium metabolism			
PTH (pg·mL^{-1})	4.0 (0.7)	3.7 (0.7)	0.22
ACa (mmol·L^{-1})	2.32 (0.05)	2.32 (0.05)	1.00
Mg (mmol·L^{-1})	0.84 (0.05)	0.83 (0.04)	0.58
PO_4 (mmol·L^{-1})	1.22 (0.12)	1.23(0.17)	0.81
Regulatory hormones			
Sclerostin (ng·mL^{-1})	0.46 (0.13)	0.46 (0.11)	0.66
IGF-1 (mmol·L^{-1})	223.2 (58.4)	235.0 (65.4)	0.37
T_3 (mmol·L^{-1})	1.66 (0.24)	1.66 (0.18)	0.86
Leptin (ng·mL^{-1})	8.3 (11.0)	11.1 (16.1)	0.21
Insulin (pmol·L^{-1})	36.1 (12.9)	44.2 (25.8)	0.18
GLP-2 (ng·mL^{-1})	10.5 (7.7)	9.4 (5.6)	0.06
Reproductive hormones			
17-*β* oestradiol (pmol.L^{-1})[2,3]	125.5 (53.2)	121.0 (46.6)	0.82

Values are expressed as means (1SD).
[1] Baseline measurements were taken on D4 in each experimental condition.
[2] Analysis performed in 10 participants with complete data for both conditions.
[3] Baseline measurements were taken on D5 in each experimental condition.
*denotes a significant difference between CON and RES (P<0.05).
DEI: Dietary energy intake; EE: Energy expenditure; *β*-CTX: C-terminal cross-linked telopeptide of type I collagen; P1NP: Amino-terminal pro-peptide of type 1 procollagen; BT ratio: Bone turnover ratio; 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; BTM: Bone turnover markers; BASE: Baseline; CON: Controlled; RES: Restricted.

4.3.3. Baseline Energy Status and Reproductive function

There were no differences in body mass, habitual DEI or lifestyle EE prior to CON and RES (Table 4.3.). Women began each condition in the early follicular phase, as confirmed by

17β-oestradiol measurements on Day 5 in both experimental conditions (Table 4.5.) (Stricker et al., 2006). At D9, *17β*-oestradiol measurements for all participants (Table 4.5.) were within the range for early (1-9 days: 5th percentile 77.99, 95th percentile: 266.08) or late follicular phase (10-14 days; 5th percentile 195.43, 95th percentile: 1146.91) (Stricker et al., 2006). One participant had slightly elevated 17*β*-oestradiol concentrations, which could denote ovulation according to Stricker et al., 2006. However, as there is some overlap in *17β*-oestradiol between phases (Stricker et al., 2006), and so analyses were conducted with and without this participant included. When this participant was excluded, there was no change in any of the BTM findings and therefore, we included the data of this participant in final analyses.

4.3.4. Compliance

The analysis of dietary plans completed by participants and/or verbal confirmation of dietary adherence on a daily basis suggest that participants complied well with the experimental diets. The actual experimental CON and RES DEI and exercise characteristics (Table 4.4.) were not different from the prescribed experimental diets in CON and RES (Table 4.1.) (data not shown, all P values>0.05). Some participants had difficulty in performing the exercise protocol in RES, although all of them were able to complete the running sessions and expended the prescribed EEE (Table 4.4).

Table 4.4. Actual experimental dietary and exercise characteristics (n=11)

	CON	**RES**
Actual Experimental Dietary characteristics		
DEI (kcal· d^{-1})	2465 (299)	1261 (125)
DEI (kcal·kg^{-1} LBM·d^{-1})	59.4 (1.4)	30.5 (0.3)
Carbohydrate (%)	49 (8)	51 (5)
Protein (%)	17 (5)	16 (4)
Fat (%)	34 (7)	32 (4)
Actual Experimental Exercise		
EEE (kcal· d^{-1})	616 (74)	616 (74)
EEE (kcal·kg^{-1} LBM·d^{-1})	14.8 (0.2)	14.8 (0.2)
Running speed (km·h^{-1})	9.0 (1.6)	9.0 (1.6)
Duration (min)	65.6 (3.9)	65.6 (3.9)

Values are expressed as means (1SD).
DEI: Dietary Energy Intake; EEE: exercise energy expenditure; LBM: Lean body mass; CON: Controlled; RES: Restricted.

4.3.5. Body mass

Body mass was maintained in CON (D4: 59.9±6.5 kg, D9:59.7± 6.2 kg, P=0.15), but significantly decreased from D5 in RES (D4: 60.2±6.20; D9: 58.6±5.9, P<0.001).

4.3.6. BTMs

4.3.6.1. *β*-CTX

There was a significant main effect of time for *β*-CTX (P=0.018), with higher mean concentrations at D7 and D9 compared to BASE. No main effect of condition (P=0.17) or an interaction effect (P=0.36) were shown (Figure 4.2.; Table 4.5.). *β*-CTX AUC was higher in RES compared to CON (P=0.033) (Figure 4.3.; Table 4.6.).

4.3.6.2. P1NP

There was a main effect of time for P1NP (P<0.001), with reduced mean concentrations at D5, D7 and D9 compared to BASE and lower concentrations at D7 compared to D5. There was no significant main effect of condition (P=0.20) or any condition x time interaction effect (P=0.21) (Figure 4.2.; Table 4.5.). P1NP AUC was lower in RES compared to CON (P=0.012) (Figure 4.3.; Table 4.5.).

4.3.6.3. BT ratio

A significant time main effect (BASE-D5; BASE-D7; BASE-D9; D5-D7; P<0.05) and a condition x time interaction effect (P=0.031) were shown for BT ratio. BT ratio was significantly reduced by 17.8% at D5, 26.2% at D7 and 25.3% at D9 relative to BASE in RES only (all P<0.05) (Figure 4.2.; Table 4.5.). BT Ratio AUC was significantly lower after RES trial (P=0.006) (Figure 4.3.; Table 4.5.).

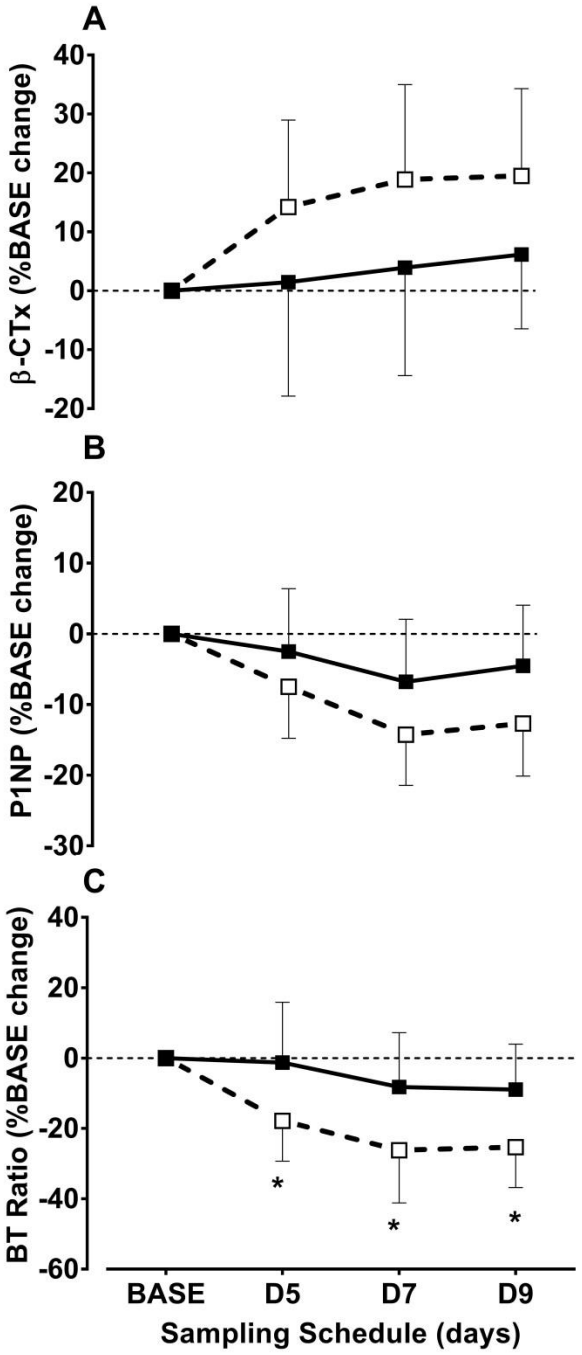


Figure 4.2. Percentage change from BASE concentrations for *β*-CTX (A), P1NP (B) and BT ratio (C) on D5-D9 in CON (black squares) and RES (white squares). Values are presented as mean (1SD). *denotes a significant difference from BASE (P<005). *β*-CTX: C-terminal cross-linked telopeptide of type I collagen; P1NP: Amino-terminal pro-peptide of type 1 procollagen; BT ratio: Bone turnover ratio; BASE: Baseline; CON: Controlled trial; RES: Restricted trial.

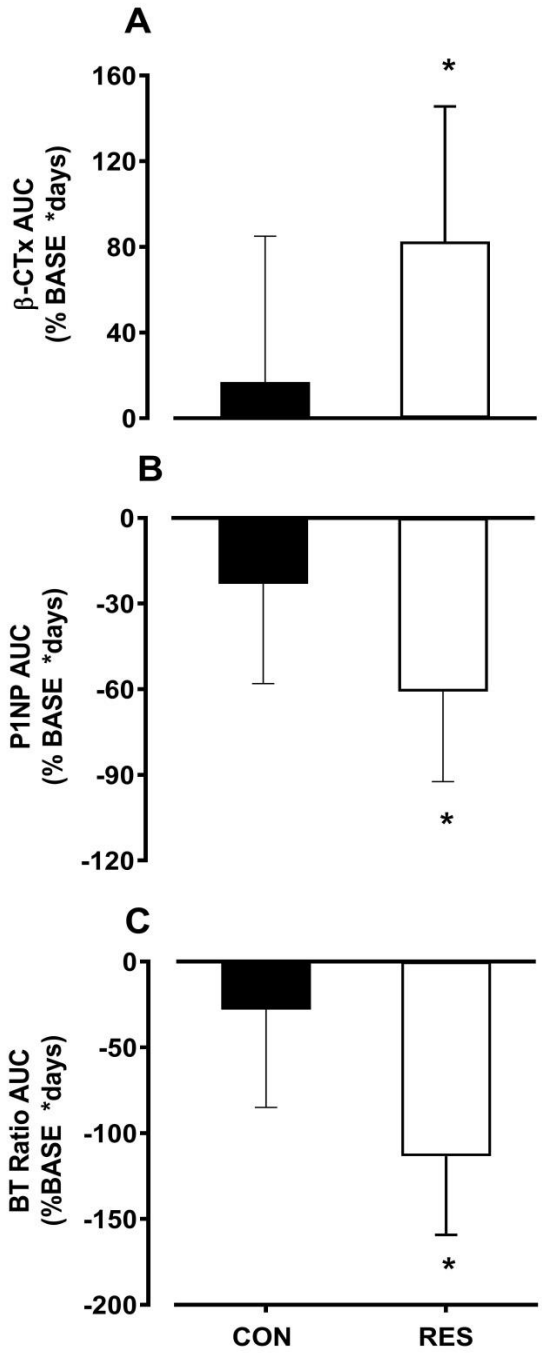


Figure 4.3. AUC analysis of *β*-CTX (A), P1NP (B) and BT ratio (C) in women after CON (black bars) and RES (white bars). Values are presented as mean (1SD). *denotes a significant difference from CON (P<0.05). *β*-CTX: C-terminal telopeptides of type I collagen; P1NP: Amino-terminal pro-peptide of type 1 procollagen; BT ratio: Bone turnover ratio; CON: Controlled trial; RES: Restricted trial; BASE: Baseline; AUC: Area under the curve.

Table 4.5. BTMs concentrations expressed as percentage change from BASE and AUC in CON and RES trials. Mean values of D1 and D3 were used as BASE prior to each experimental condition.

	CON		RES	
	BASE	**D9**	**BASE**	**D9**
***β*-CTX**				
$\mu g \cdot L^{-1}$	0.49 (0.14)	0.51 (0.12)	0.42 (0.11)	0.49 (0.09)
%BASE change		6.1 (12.6)		19.5 (14.8)
AUC (%BASE x d)[1]		16.9 (68.1)		85.7 (60.5)**
P1NP				
$\mu g \cdot L^{-1}$	71.1 (15.0)	67.5 (13.7)	70.6 (15.1)	61.6 (14.0)
%BASE change		-4.5 (8.6)		-12.7 (7.4)
AUC (%BASE x d)[1]		-23.1 (34.9)		-60.9 (31.2)**
BT ratio				
-	1.54 (0.44)	1.39 (0.39)	1.73 (0.42)	1.27 (0.33)*
%BASE change		-9.0 (12.9)		-25.3 (11.4)
AUC (%BASE x d)[1]		-28.0 (56.9)		-113.4 (45.8)**

Values are expressed as means (1SD).
[1]AUC calculated for each experimental condition from BASE to D9.
*denotes a significant difference from BASE in the same condition (P<0.05).
**denotes a significant difference from CON at the same timepoint (P<0.05).
β-CTX: C-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; D: Day; BASE: Baseline; AUC: Area under the curve.

4.3.7. Markers of calcium metabolism

4.3.7.1. PTH

There was a significant main effect of time (P=0.009), with levels at D7 increasing from BASE (P=0.003). There was no significant main effect of condition (P=0.62) or any condition x time interaction effect (P=0.60). PTH AUC in RES was not significantly different from AUC in CON (P=0.39) (Table 4.6.).

4.3.7.2. ACa

There was no significant main effect of time (P=0.69), condition (P=0.69) or any condition x time interaction effect (P=0.45) was shown for ACa levels. ACa AUC was not significantly different between CON and RES (P=0.42) (Table 4.6.).

4.3.7.3. Mg

There was no significant main effect of time (P=0.17), condition (P=0.98) or any condition x time interaction effect (P=0.69) was shown for Mg levels. Mg AUC was not significantly different between CON and RES (P=0.18) (Table 4.6.).

4.3.7.4. PO_4

There was no significant main effect of time (P=0.25) or condition (P=0.92). A condition x time interaction (P=0.02) effect was shown for PO_4 concentrations, although no *post-hoc* comparisons reached significance. PO_4 AUC was not significantly different between CON and RES (P=0.93) (Table 4.6.).

Table 4.6. Markers of calcium metabolism expressed as concentrations, percentage change from BASE and AUC in CON and RES trials. Mean values of D1 and D3 were used as BASE prior to each experimental condition.

	CON		RES	
	BASE	**D9**	**BASE**	**D9**
PTH				
$pg \cdot mL^{-1}$	3.98 (0.66)	4.18 (0.95)	3.68 (0.69)	4.26 (1.03)
%BASE change		4.6 (17.6)		13.6 (17.9)
AUC (%BASE x d)[1]		49.9 (59.4)		73.9 (82.9)
ACa				
$mmol \cdot L^{-1}$	2.32 (0.05)	2.33 (0.06)	2.32 (0.05)	2.32 (0.04)
%BASE change		0.8 (1.8)		0.0 (1.6)
AUC (%BASE x d)[1]		-0.9 (5.9)		1.0 (6.8)
Mg				
$mmol \cdot L^{-1}$	0.84(0.05)	0.84(0.04)	0.83 (0.04)	0.85 (0.05)
%BASE change		0.8 (2.7)		2.0 (4.4)
AUC (%BASE x d)[1]		2.5 (17.6)		10.1 (21.3)
PO_4				
$mmol \cdot L^{-1}$	1.22 (0.12)	1.30 (0.17)	1.23 (0.17)	1.23 (0.13)
%BASE change		7.3 (6.7)		1.0 (8.4)
AUC (%BASE x d)[1]		16.9 (36.9)		18.8 (36.1)

Values are expressed as means (1SD).
[1]AUC calculated for each experimental condition from BASE to D9.
PTH: Parathyroid hormone; Mg: Magnesium; ACa: Albumin adjusted Calcium; PO_4: Phosphate; D: Day; BASE: Baseline; CON, Controlled trial; RES, Restricted trial.

4.3.8. Regulatory hormones

4.3.8.1. Sclerostin

There was no significant main effect of time (P=0.14), condition (P=0.97), or any condition x time interaction effect (P=0.67) for sclerostin concentrations. Sclerostin AUC in CON did not significantly differ from that in RES (P=0.48) (Table 4.7.).

4.3.8.2. IGF-1

There was a significant main effect of time (P<0.001), with mean IGF-1 concentrations decreasing progressively from BASE to D9 (BASE-D5; BASE-D7; BASE-D9; D5-D7; D5-

D9; all P<0.001). There was no significant main effect of condition (P=0.76) or any condition x time interaction effect (P=0.22). IGF-1 AUC was not significantly different between conditions (P=0.09) (Table 4.7.)

4.3.8.3. T_3

There was no significant main effect of condition (P=0.08) or time (P=0.26). A time x condition interaction was shown (P=0.006), with mean T_3 levels at D9 being significantly reduced by 10.7% from BASE (P=0.008) in response to RES, but not to CON. T_3 AUC in RES was not significantly different from that in CON (P=0.054) (Table 4.7.).

4.3.8.4. Insulin

A significant main effect of condition (P=0.012) and time (P=0.007) and a condition x time interaction effect (P<0.001) were shown for insulin concentrations. Mean insulin concentrations at D7 were significantly reduced from BASE (P=0.005). In response to RES, insulin concentrations were lower at D7 (-45.3% from BASE and -24.3% from D5) and at D9 (-29.2% change from BASE). Insulin AUC after RES was lower than after CON (P=0.011) (Table 4.7.).

4.3.8.5. Leptin

Mean leptin levels were lower between BASE-D5, BASE-D7, BASE-D9 and D5-D7 (time effect; P <0.001). There was no significant main effect of condition (P=0.21) or any time x condition interaction effect (P=0.054). Leptin AUC was significantly lower after RES compared to CON (P=0.043) (Table 4.7.).

4.3.8.6. GLP-2

There was no significant main effect of time (P=0.30) or condition (P=0.85) or any time x condition interaction effect (P=0.60). GLP-2 AUC was not significantly different between RES and CON (P=0.14) (Table 4.7.).

4.3.9. Reproductive hormones

There was no significant main effect of time (P=0.053) or condition (P=0.83) or any condition x time interaction effect (P=0.53) for 17β-oestradiol levels. 17β-oestradiol AUC was not significantly different between conditions (P=0.39) (Table 4.7.).

Table 4.7. Regulatory and reproductive hormones expressed as concentrations, percentage change from BASE and AUC in CON and RES trials. Mean values of D1 and D3 were used as BASE prior to each experimental condition.

	CON		RES	
	BASE	**D9**	**BASE**	**D9**
Sclerostin				
ng·mL^{-1}	0.45 (0.13)	0.47 (0.12)	0.46 (0.11)	0.49 (0.13)
%BASE change		6.8 (17.3)		4.5 (8.8)
AUC (%BASE x d)[1]		-78.2 (64.9)		-7.1 (39.2)
IGF-1				
mmol·L^{-1}	223.2(58.4)	202.0 (57.8)	235.0 (65.5)	186.1 (57.5)
%BASE change		-8.8 (20.9)		-20.0 (9.6)
AUC (%BASE x d)[1]		-48.2 (82.6)		-89.8 (47.5)
T_3				
mmol·L^{-1}	1.65 (0.25)	1.66 (0.22)	1.66 (0.19)	1.47 (0.18)*
%BASE change		1.9 (7.0)		10.7 (10.2)
AUC (%BASE x d)[1]		-1.8 (61.3)		-31.5 (40.5)
Leptin				
ng·mL^{-1}	8.3 (11.0)	5.9 (5.7)	11.1 (16.1)	4.3 (5.0)
%BASE change		-8.8 (34.9)		-39.8 (38.5)
AUC (%BASE x d) [1]		-118.3 (119.0)		-214.2 (112.2)**
Insulin				
pmol·L^{-1}	36.1 (12.9)	37.3 (15.1)	44.2 (25.8)	22.4 (13.0)*
%BASE change		19.2 (57.9)		-29.2 (47.3)
AUC (%BASE x d) [1]		-16.5 (194.2)		-171.8 (121.7)**
GLP-2				
ng·mL^{-1}	10.5 (7.7)	9.5 (4.9)	9.4 (5.6)	9.8 (6.8)
%BASE change		-4.0 (17.8)		3.7 (16.0)
AUC (%BASE x d) [1]		-20.2 (70.5)		11.5 (46.3)
17-β oestradiol				
pmol.L^{-1}	115.5(45.4)	186.4 (86.1)	126.0 (46.5)	226.9 (200.8)
%BASE change		59.5 (54.3)		39.3 (31.5)
AUC (%BASE x d) [1]		119.0 (108.7)		78.5 (63.0)

Values are expressed as means (1SD).
[1]AUC calculated for each experimental condition from BASE to D9.
[2]ANOVA performed in 9 participants with available data in both conditions.
*denotes a significant difference from BASE in the same condition (P<0.05).
**denotes a significant difference from CON at the samepoint (P<0.05).
T_3: Triiodothyronine; IGF-1: Insulin-like growth factor 1; GLP-2: Glucagon-like peptide 2, D: Day; BASE: Baseline; CON, Controlled trial; RES, Restricted trial.

4.3.10. Individual analysis

Individual responses were considered in line with the criteria outlined in section 4.2.6. Seven out of 11 participants responded to RES with an increase in β-CTX concentrations and 6 out of 11 participants responded to RES with a decrease in P1NP concentrations. Four female participants were responders for β-CTX (increase) only, 3 participants were responders for P1NP (decrease) only and 3 participants responded to RES for both. In total, 10 out 11

women had altered bone turnover resulting from changes in bone resorption (increase), bone formation (decrease) or both (Table 4.8.).

Table 4.8. 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.

	***β*-CTX**	**P1NP**	**Bone turnover[1]**	**Bone turnover[2]**
RES	7/11	6/11	10/11	3/11

[1]altered bone turnover due to increase *β*-CTX, decreased P1NP or both.
[2]altered bone turnover due to a simultaneous increase in *β*-CTX and decrease in P1NP.
β-CTX: C-terminal cross-linked telopeptide of type I collagen; P1NP: Amino-terminal pro-peptide of type 1 procollagen; BT ratio: Bone turnover ratio; BASE: Baseline; RES: Restricted.

4.4. Discussion

This study is the first to investigate short-term, low EA at 15 kcal·kgLBM^{-1}·d^{-1} on BTMs, regulatory hormones, markers of calcium metabolism and reproductive hormones in physically active women. The main findings were that low EA over a short-time period of 5 days resulted in a significant increase in bone resorption (β-CTX AUC) and a significant decrease in bone formation (P1NP AUC). These changes were accompanied by adaptive changes in regulatory hormones including insulin, leptin and T_3, which were indicative of energy conservation.

These findings extend those of previous, short-term, laboratory studies documenting a decrease in bone formation, with or without an increase in bone resorption, with low EA (Ihle and Loucks, 2004) and acute fasting (Grinspoon et al., 1995) in sedentary women. The increases in bone resorption (β-CTX: +19%) shown at 15 kcal·kgLBM^{-1}·d^{-1} occur in the same direction as the changes documented at 10 kcal·kgLBM^{-1}·d^{-1} (urinary NTX: +34%) by Ihle & Loucks (2004). 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 (Ihle & Loucks, 2004). 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.* P1CP) and in different samples (*i.e.,* plasma and urine). In addition to these differences, the dietary prescriptions and training status of participants were not similar between these studies. The results of Ihle and Loucks (2004) suggest that changes occur in untrained women, under different levels of energy deficit, if they follow a short-term training programme. The current study was conducted in physically active women whose bone would be used to the demands of weight-bearing exercise, with the level of low EA chosen to represent the EA of amenorrheic athletes, who are at high risk for bone injuries (Thong et al., 2000), making the current results relevant to regular exercisers.

Even slight changes in bone formation and resorption may increase the risk for impaired bone turnover, as suggested by a 24% reduction in BT ratio in response to RES, but not CON. Individual analysis showed a consistent bone turnover response, with 10 out of the 11 participants responding to RES with changes in bone resorption (increase), bone formation (decrease) or both. Taken together, these bone turnover responses suggest a net bone loss, which if left uncontrolled, could unfavourably influence bone health and increase the risk of bone injury. Such alterations might manifest via changes in bone volume, mineralisation of

the bone matrix, collagen cross-linking and the appearance of remodelling cavities that may act as stress inducers (Hernadez, 2008).

We did not show any alteration in sclerostin levels in low EA compared to controlled EA. Sclerostin is secreted by osteocytes and acts as a Wnt antagonist that, through the Wnt/β-catenin pathway, regulates osteoblast activity (Robling et al., 2008) and, therefore, bone formation. The absence of changes in sclerostin following RES in our female participants suggests that the observed reduction in P1NP is not mediated by sclerostin. Sclerostin is highly responsive to changes in mechanical loading (Spatz et al, 2013; Belavy et al, 2016) and exercise during weight loss prevents the increase in sclerostin (Armamento-Villareal et al, 2012), 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 the controlled and restricted EA.

The precise mechanisms through which EA may elicit its effect on bone turnover have not been elucidated, but may involve changes in energy regulatory hormones (Ihle & Loucks., 2004; Misra & Klibanski, 2014; Shapses & Sukumar, 2012), reproductive hormones (Misra & Klibanski, 2014; Fazeli & Klibanski, 2014; De Souza et al., 2008) or markers of calcium metabolism (Scott et al., 2012; Haakonssen et al., 2015). Women demonstrated hormonal changes indicative of energy conservation with decreases in leptin, insulin, and T_3, which are in agreement with previous studies investigating energy deprived conditions (Ihle & Loucks, 2004; De Souza et al., 2008). These results are in line with the well-established actions of these hormones to reverse energy deficit and preserve energy stores (Misra & Klibanski, 2011; 2012). Leptin exerts direct and indirect actions on bone metabolism that are generally protective of BMD (for review see Upadhyay et al., 2015). Insulin is a potential determinant of BMD (Haffner & Bauer,1993) and insulin deficiency is often accompanied by reduced BMD and increased fragility risk (Misra et al, 2007; Nyman et al, 2011), although this seems to have an effect on specific bones 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 (Huang et al, 2000; Lakatos, 2000), although the latter seems unlikely in the current study given that there was no effect of RES on IGF-1. It is possible that a suppression of IGF-1 was masked by an increase in circulating concentrations of binding proteins (Fontana et al., 2016), which were not determined in the present study.

In the current study, 5 days of low EA did not influence 17-β oestradiol levels. Previous short-term energy restriction experiments have indicated that changes in sex hormones

(Loucks et al., 1998) are manifested over a more prolonged period (>1 week), despite early reductions in LH pulsatility (Loucks & Thuma, 2003). Another possible reason for our result may be that the level of reduced EA was not severe enough to suppress 17-β oestradiol levels. Subtle reductions in oestradiol levels have been shown at 10 kcal·kgLBM^{-1}·d^{-1} and were correlated with increased bone resorption (Ihle & Loucks, 2004). It was long believed that hypogonadism is a major determinant of low BMD in women with anorexia nervosa and amenorrheic athletes (Drinkwater, 1984; 1990). Irregular menstrual cycles (Maimoun et al., 2014), delayed menarche (Maimoun et al., 2014) and use of OCPs (Polatti et al., 1995; Nappi et al., 2003) have been associated with lower-than-optimal peak bone mass attainment, impaired bone turnover, low BMD, altered microarchitecture and bone fragility. Evidence suggests that hypogonadism is important, but not the only factor responsible for bone loss in energy-deficient states (Fazeli & Klibanski, 2014; De Souza et al., 2008). Low BMDs in anorexia nervosa patients have not been fully normalised by either oestrogen treatment (Klibanski et al., 1995) or after menses recovery (Misra et al., 2008).

Another possible explanation for the effects of low EA on bone metabolism is the change in makers of calcium metabolism. The present study did not reveal any effects of low EA on PTH, ACa, Mg or PO_4 levels, thus, it is unlikely that the alterations on bone metabolism in the present study were mediated by changes in these markers. PTH is the major regulator of calcium homeostasis. Low levels of extracellular ionised calcium trigger an increase in PTH release that mobilise intracellular calcium through PTH/PTH-related peptide receptor-dependent mechanisms which activate G protein signals (Poole & Reeve, 2005). These results are unsurprising, since participants were provided a multi-mineral supplement during the restricted trial to isolate the effects of energy restriction. It is worth noting that energy/macronutrient restriction is commonly accompanied by reduction in the vitamins and minerals necessary for bone health (Shapses & Sukumar, 2012) and future studies should separate the effects of macronutrient from micronutrient restriction on bone metabolism.

To conclude, short-term low EA at 15 kcal·kgLBM^{-1}·d^{-1} decreased bone formation and increased bone resorption in physically active, eumenorrheic women. These changes were accompanied by significant reductions in regulatory hormones; leptin, insulin and T_3, but no changes in reproductive hormones or markers of calcium metabolism were shown. Our findings support previous studies showing that low EA underpins bone health in women (Nattiv et al., 2007; De Souza *et al.*, 2014a; Mountjoy et al., 2014). Further studies are necessary to determine whether the observed changes are preserved over time.

Chapter 5. Bone metabolic response to short-term low energy availability in physically active men

5.1. Introduction

Reduced EA in sport has been predominantly seen as a women's issue (Nattiv et al., 2007; De Souza et al., 2014a). Emerging evidence stemming from the RED-S model suggests that men also adopt low EA; with those participating in weight sensitive sports being at a greater risk (Mountjoy et al., 2014; Tenforde et al., 2016). Similar to female athletes, male athletes may undertake high training volumes (Julian-Almarcegui et al., 2013; Vogt et al., 2005; Pontzer et al., 2015) and/or restrict their DEI unintentionally or deliberately in order to achieve specific weight and body composition goals (Loucks, 2007; Loucks et al., 2011). As a result, moderately to severely restricted EAs have been reported in male athletes, including Kenyan runners (approximately 35 kcal·kgLBM^{-1}·d^{-1}) (Loucks et al., 2011), jump jockeys (approximately 19 kcal·kgLBM^{-1}·d^{-1}) (Wilson et al., 2013; 2014) and cyclists training for the Tour de France (8 kcal·kgLBM^{-1}·d^{-1}) (Vogt et al., 2005).

Low EA has been associated with reproductive disturbances and impaired health in women, which is known collectively as the Female Athlete Triad (Nattiv et al, 2007; De Souza et al., 2014a). The RED-S models introduced by the IOC (Mountjoy et al., 2014) and other recent reviews (Tenforde et al., 2016) have paralleled these conditions in men, suggesting that some male athletes may also be prone to low EA and experience skeletal disorders. Low BMD including both osteopenia and osteoporosis (Hetland et al., 1993; Hind et al., 2006; Olmedillas et al., 2012; Fredericson et al., 2007), altered bone turnover (Dolan et al., 2012; Hetland et al., 1993) and a higher risk for a stress fracture injury (Kussman et al., 2015; Nattiv et al., 2013) have been reported in males with low EA and other risk factors associated with low EA (*i.e.*, low BMI, disordered eating). Despite the identification of the co-existence of low EA and impaired bone health in male athletic subgroups; the characteristics, prevalence, clinical significance and association between these conditions remain to be determined.

The effects of low EA on bone metabolic responses have been explored in sedentary women (Ihle & Loucks, 2004). In Study 1 (reported in Chapter 4), markers of bone formation were reduced, whereas markers of bone resorption were increased following EA at 15 kcal·kgLBM^{-1}·d^{-1} in physically active women. Current understanding on the effects of low EA on bone metabolism and health in men is limited. Zanker and Swaine (2000) examined the effect of exercise and/or reduced food intake on bone metabolism in trained men, showing a reduction in bone formation, without an effect on bone resorption (Zanker and Swaine, 2000). In their study, however, energy restriction was determined using energy balance rather than EA. The estimation of TEE required for energy balance determination

may introduce more sources of errors than the measurement of EEE, which is only required for determining EA. Additionally, energy balance formulas accept that bodily systems function normally, however, increases in EEE may cause suppression of bodily functions (Loucks, 2013). In this case, individuals may be in energy balance but experience low EA (Stubbs et al., 2004; Loucks 2007; 2013). As such, well-controlled experimental studies are needed to characterise the relationship between low EA and bone turnover in this population.

As shown in the previous study, outlined in Chapter 4, low EA has been associated with reductions in the concentration of energy regulatory hormones such as leptin, insulin and T_3 in women. Others have also shown similar findings at EAs of less than 30 kcal·kgLBM^{-1}·d^{-1} (Loucks & Thuma, 2003; Ihle and Loucks, 2004). Low EA also results in the suppression of GnRH, LH pulsatility and oestrogens in women (De Souza et al., 2004; Loucks & Thuma, 2003). The independent or synergistic action of these factors might mediate bone turnover responses in the affected women (De Souza et al., 2008; Ihle & Loucks, 2004). The contribution of hormonal changes due to low EA on bone turnover in men needs to be determined. Suppression of T_3, leptin, IGF-1 and testosterone have been previously shown in male soldiers exposed to energy deficits of various magnitudes during military training (Kyrolainen et al., 2008; Friedl et al., 2000; Hoyt & Friedl, 2006).

To address these issues, we conducted a randomised, crossover study to explore the effects of short-term low EA at 15 kcal·kgLBM^{-1}·d^{-1} on BTM in physically active men. Low EA was achieved by dietary energy restriction and EEE. Alterations in regulatory hormones and markers of calcium metabolism were also explored, as possible mediating mechanisms to the bone metabolic outcomes. It was hypothesised that bone formation would decrease and bone resorption would be increase in response to low EA.

5.2. Methods

5.2.1. Participants

Eleven men volunteered to participate in the study (Table 5.1.). Before inclusion, all participants provided verbal and written informed consent. Inclusion and exclusion criteria were identical with those applied in Study 1 with the exception of those criteria regarding the reproductive function for women. Please see section 4.2.1.

5.2.2. Experimental design

The experimental design was identical to that of Study 1 (reported in Chapter 4). A schematic representation and a detailed description of experimental design can be found in section 4.2.2.

5.2.3 Experimental procedures

The experimental procedures were identical to those of Study 1 (reported in Chapter 4). Detailed description has been provided for preliminary assessment in section 4.2.3.1., experimental period in section 4.2.3.2., lifestyle EE in section 4.2.3.2.1., habitual DEI in section 4.2.3.2.2., experimental diets in section 4.2.3.2.3., EEE in section 4.2.3.2.4 and compliance to experimental condition in section 4.2.3.2.5.

Identical to the study in women, during D4-D8, male participants were given diet plans containing the same foods and beverages consumed during the 3-days of their habitual diet in amounts that provided dietary energy intake of 60 and 30 $kcal \cdot kg^{-1}LBM \cdot d^{-1}$ and maintained the dietary composition of each participant's habitual diet. Table 5.2 provides summary information for energy and macronutrient compositions of prescribed experimental diets in this study in men.

Table 5.2. Energy and macronutrient composition of prescribed experimental diets.

	CON	RES
DEI (kcal· d^{-1})	3431 (348)	1722 (210)
DEI (kcal·kg^{-1} LBM·d^{-1})	59.9 (0.2)	30.1 (0.2)
Carbohydrate (%)	47 (9)	48 (9)
Protein (%)	19 (4)	19 (4)
Fat (%)	35 (7)	34 (7)

Values are expressed as means (1SD).
DEI: Dietary Energy Intake; LBM: Lean Body Mass; CON: Controlled; RES: Restricted.

5.2.4. Storage and analyses of blood samples

Blood samples were collected and analysed as previously described in section 4.2.4.

5.2.5. Biochemical analysis

Biochemical analysis has been previously described in section 3.13.; with the exception of 17β-oestradiol concentration that was not determined in men.

5.2.6 Statistical analysis

Based on results reported by Zanker and Swaine (2000), the study was sized 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. A *priori* power calculations showed that a minimum of 7 men were required to achieve 95% power at $P<0.05$. Statistical analysis was performed as previously described in section 4.2.6. Individual analysis of the BTM data was performed in an identical way as in the women's study (Chapter 4). Criteria for responders and non-responders to RES for β-CTX and P1NP have been established in section 4.2.6.

5.3. Results

5.3.1. Participant characteristics

Participant characteristics are presented in Table 5.2.

Table 5.1. Participant characteristics (n=11)

Demographics	
Age (y)	26 (5)
Height (m)	1.78 (0.07)
Body mass (kg)	73.0 (8.0)
BMI (kg·m^2)	23.0 (1.6)
Body composition	
Body fat (%)	18.3 (3.4)
LBM (kg)	57.2 (7.3)
FFM (kg)	60.3 (7.6)
BMD (g·cm^{-2})	1.25 (0.08)
Training characteristics	
VO_2 peak (ml·kg^{-1}·min^{-1})	54.2 (5.3)
Physical activity (min·d^{-1})	57.9 (29.0)
Physical activity (MET-min·wk^{-1})	3443 (1006)
Dietary and EE characteristics	
Habitual DEI (kcal·d^{-1})	2682 (265)
Lifestyle EE (kcal·d^{-1})	449 (136)
24-hour EE(kcal· d^{-1})	2398 (185)

Values are expressed as means (1SD).
[1] Mean values of D1-D3 prior to both experimental conditions.
BMI: Body mass index; LBM: Lean body mass; FFM: Fat free mass; BMD: Bone mineral density: VO_{2max}: Maximum oxygen uptake; DEI: Dietary energy intake; EE: Energy expenditure; RMR: Resting metabolic rate; LBM: Lean body mass; MET: Metabolic equivalents.

5.3.2. Baseline biochemistry

Baseline leptin before RES was significantly higher than baseline levels in CON (P=0.01), but there were not any further differences in any other biochemical marker (Table 5.3.). Baseline concentrations for BTM were towards the higher end of the reference range (β-CTX: 0.17-0.60 µg·L^{-1}; P1NP: 15-80 µg·L^{-1}; Jenkins et al., 2013) for both β-CTX and P1NP (Table 5.3.).

Table 5.3. Markers of energy status and BASE concentrations of BTMs and markers of calcium metabolism, regulatory prior to CON and RES trials. Mean values of D1 and D3 were used as BASE prior to each experimental condition (n=11).

	CON	RES	P-value
Markers of energy status			
Body mass (kg)[1]	72.7 (8.1)	73.0 (8.1)	0.26
Habitual DEI (kcal·d^{-1})	2697 (303)	2667 (289)	0.72
Lifestyle EE (kcal·d^{-1})	451 (138)	447 (177)	0.94
BTMs			
***β*-CTX (µg·L^{-1})**	0.48 (0.16)	0.50 (0.16)	0.44
P1NP (µg·L^{-1})	73.6 (31.4)	74.8 (28.7)	0.75
BT ratio	1.56 (0.46)	1.51 (0.40)	0.64
Markers of calcium metabolism			
PTH (pg·mL^{-1})	2.79 (1.14)	2.93 (1.0)	0.41
ACa (mmol·L^{-1})	2.39 (0.06)	2.35 (0.06)	0.06
Mg (mmol·L^{-1})	0.83 (0.05)	0.83 (0.05)	0.87
PO_4 (mmol·L^{-1})	1.19 (0.12)	1.25 (0.16)	0.32
Albumin	45.1 (1.9)	44.8 (2.2)	0.42
Regulatory hormones			
Sclerostin (ng·mL^{-1})	0.61 (0.15)	0.60 (0.14)	0.71
IGF-1 (mmol·L^{-1})	176.6 (57.6)	184.7 (55.6)	0.54
T_3 (mmol·L^{-1})	1.74 (0.25)	1.83 (0.35)	0.13
Leptin (ng·mL^{-1})[2]	2.3 (1.4)	3.4 (1.8)	0.008*
Insulin (pmol·L^{-1})	33.0 (14.1)	35.1 (9.9)	0.45
GLP-2 (ng·mL^{-1})	15.0 (12.5)	14.1 (12.5)	0.36

Values are expressed as means (1SD).
[1] Baseline measurements were taken on D4 in each experimental condition.
[2] Analysis performed in 6 participants- in 5 participants, leptin levels were undetectable (<1 ng·mL^{-1}).
*denotes a significant difference between CON and RES (P<0.05).
DEI: Dietary energy intake; EE: Energy expenditure; *β*-CTX: C-terminal cross-linked telopeptide of type I collagen; P1NP: Amino-terminal pro-peptide of type 1 procollagen; BT ratio: Bone turnover ratio; 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; CON: Controlled; RES: Restricted.

5.3.3. Baseline energy status

There were no differences in habitual dietary intake, lifestyle EE or body mass prior to CON and RES (Table 5.3.).

5.3.4. Compliance

The actual experimental diets (Table 5.1.) were not different from the prescribed experimental diets in CON and RES (Table 5.4.) (data not shown, all P values >0.05), indicating that participants complied well with the experimental diets. All participants completed the running sessions and expended the prescribed EEE (Table 5.4).

Table 5.4. Actual experimental dietary and exercise characteristics (n=11)

	CON	**RES**
Actual Experimental Diets		
DEI (kcal· d^{-1})	3383 (393)	1720 (235)
DEI (kcal·kg^{-1} LBM·d^{-1})	59.2 (1.1)	30 (0.2)
Carbohydrate (%)	47 (8)	48 (9)
Protein (%)	18 (3)	19 (4)
Fat (%)	35 (7)	33 (7)
Actual Experimental Exercise		
EEE (kcal· d^{-1})	856 (110)	856 (110)
EEE (kcal·kg^{-1} LBM·d^{-1})	15.0 (0.1)	15.0 (0.1)
Running speed (km·h^{-1})	10.5 (1.7)	10.5 (1.7)
Duration (min)	65 (7)	65 (7)

Values are expressed as means (1SD).
DEI; Dietary energy Intake; EEE: Exercise Energy Expenditure; LBM: Lean Body Mass; CON: Controlled; RES: Restricted.

5.3.5. Body mass

Body mass was maintained in CON (D4: 72.7±8.1; D9: 72.9±8.1 kg, P=0.31), but significantly decreased from BASE in RES (D4: 73.0 ±8.1; D9: 71.2 ±7.9 kg; P<0.001).

5.3.6. BTMs

5.3.6.1. *β*-CTX

There was no significant effect of time (P=0.14), condition (P=0.34) or any condition x time interaction effect (P=0.61) *β*-CTX concentrations (Figure 5.1.; Table 5.5.) *β*-CTX AUC was not significantly different between CON and RES (P=0.46) (Figure 5.2.; Table 5.5.).

5.3.6.2. P1NP

There was a significant main effect of time (D9< BASE; P=0.02), but no significant main effect of condition (P=0.39) or any condition x time interaction effect (P=0.11) was shown for P1NP concentrations. (Figure 5.1.; Table 5.5.). P1NP AUC in CON was not significantly different from that in RES (P=0.12) (Figure 5.2.; Table 5.5).

5.3.6.3. BT ratio

There was a significant main effect of time (P=0.03) for BT ratio, with the ratio at D9 being significantly lower compared to BASE (P=0.02). No significant main effect of condition (P=0.31) or any condition x time interaction effect (P=0.18) was shown for BT ratio (Figure 5.1.; Table 5.5.). BT ratio AUC in CON was not different from that in RES (P=0.33) (Figure 5.2.; Table 5.5).

Table 5.5. BTMs expressed as concentrations, percentage change from BASE and AUC in CON and RES trials (n=11). Mean values of D1 and D3 were used as BASE prior to each experimental condition.

	CON		**RES**	
	BASE	**D9**	**BASE**	**D9**
***β*-CTX**				
$\mu g \cdot L^{-1}$	0.48 (0.16)	0.52 (0.17)	0.50 (0.17)	0.57 (0.26)
%BASE change		13.1 (33.3)		12.2 (16.8)
AUC (%BASE x d)[1]		60.5 (126.7)		39.0 (72.3)
P1NP				
$\mu g \cdot L^{-1}$	73.6 (31.4)	72.5 (36.7)	74.8 (28.7)	64.3 (25.3)
%BASE change		-1.76 (16.2)		-14.03 (8.0)
AUC (%BASE x d)[1]		-5.2 (75.1)		-46.6 (24.3)
BT ratio				
-	1.57 (0.46)	1.39 (0.42)	1.52 (0.40)	1.16 (0.29)
%BASE change		-6.8 (29.7)		-21.1 (16.3)
AUC (%BASE x d)[1]		-24.2 (171.0)		-66.4 (70.8)

Values are expressed as means (1SD).
[1]AUC calculated for each experimental condition from BASE to D9.
β-CTX: C-terminal cross-linked telopeptide of type I collagen; P1NP: Amino-terminal pro-peptide of type 1 procollagen; BT ratio: Bone turnover ratio; BTM: Bone turnover marker; D: Day; BASE: Baseline; CON, Controlled trial; RES, Restricted trial.

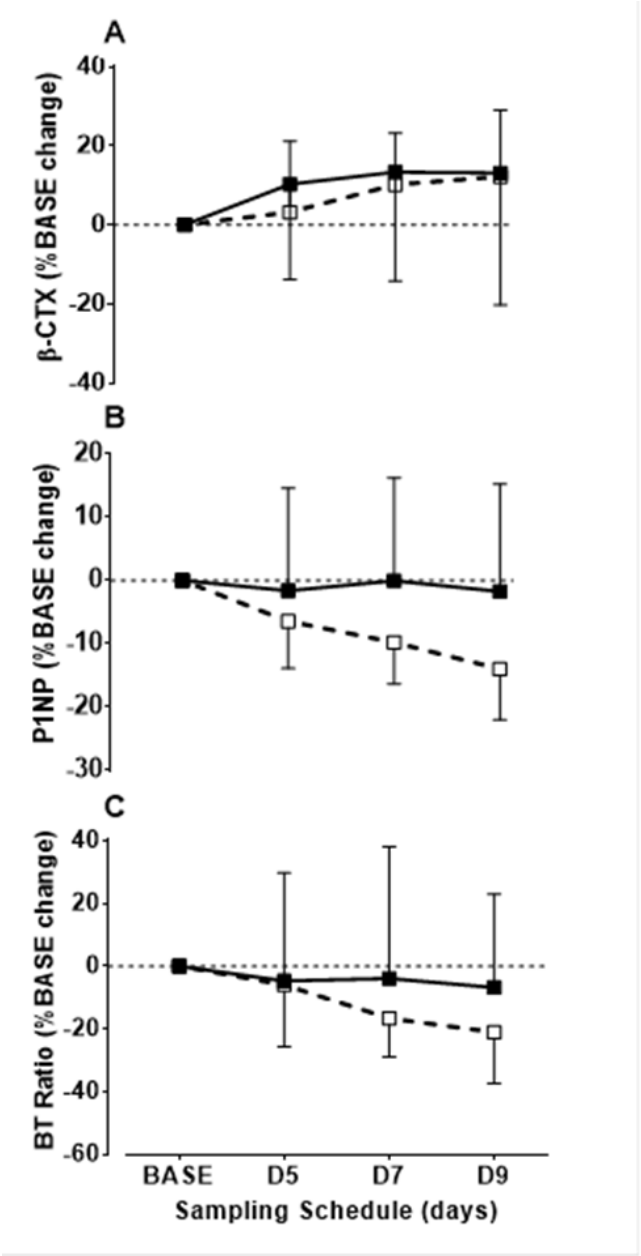


Figure 5.1. Percentage change from baseline (BASE) concentrations for β-CTX (A), P1NP (B) and BT ratio (C) on D5-D9 in CON (black squares) and RES (white squares) (n=11). Values are presented as means (1SD). *β*-CTX: C-terminal cross-linked telopeptide of type I collagen; P1NP: Amino-terminal pro-peptides of type 1 procollagen; BT ratio: Bone turnover ratio; D: Day; BASE: Baseline; CON: Controlled trial; RES: Restricted trial.

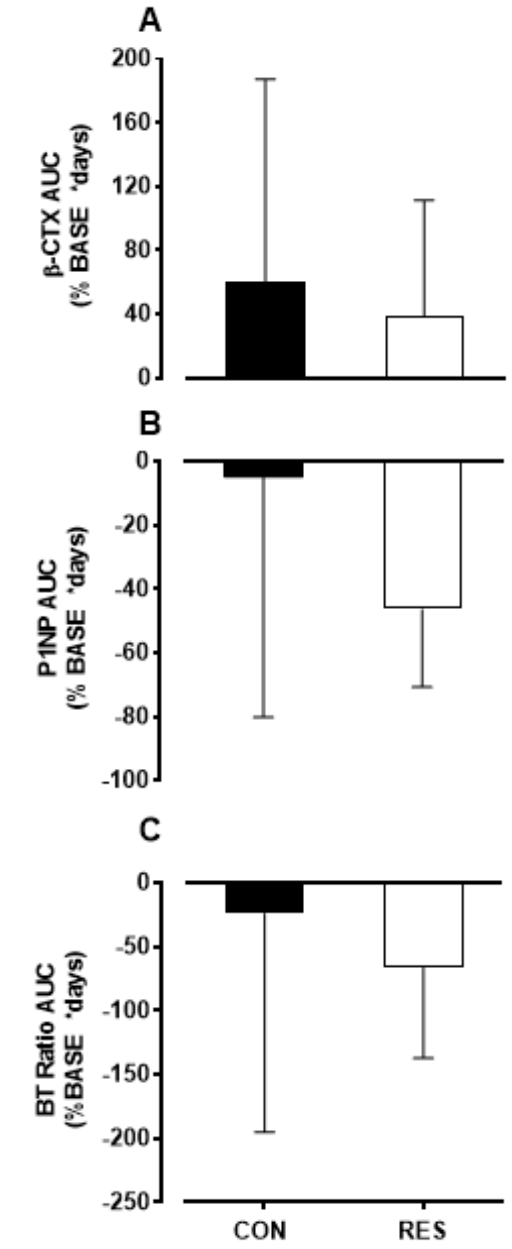


Figure 5.2. AUC analysis of β-CTX (A), P1NP (B) and BT ratio (C) after CON (black bars) and RES (white bars) (n=11). Values are presented as means (1SD). *β*-CTX: C-terminal cross-linked telopeptide of type I collagen; P1NP: Amino-terminal pro-peptides of type 1 procollagen; BT ratio: Bone turnover ratio; D: Day; BASE: Baseline; AUC: Area under the curve; CON: Controlled trial; RES: Restricted trial.

5.3.7. Markers of calcium metabolism

5.3.7.1. PTH

There was no significant main effect of time (P=0.59), condition (P=0.79) or any condition x time interaction effect for PTH concentrations P=0.39). PTH AUCs were not significantly different between CON and RES (P=0.11) (Table 5.6.).

5.3.7.2. ACa

There was a significant main effect of time (D9<BASE, P=0.01; D9<D5, P=0.01), ACa levels were lower in RES than CON (P=0.046), which may be in part due to baseline changes prior to CON and RES (RES<CON). There was no significant time x condition interaction effect (P=0.14). ACa AUC was not not significantly different by condition (P=0.09) (Table 5.6.).

5.3.7.3. Mg

There were no significant main effects of time (P=0.70) or condition (P=0.46) and no significant condition x time interaction effect was shown for Mg levels (P=0.59). Mg AUCs were not significantly different between CON and RES (P=0.11) (Table 5.6.).

5.3.7.4 PO_4

There was no significant main effect of time (P=0.24), condition (P=0.85) or any condition x time interaction effect (P=0.18) for PO_4 levels. PO_4 AUC did not significantly differ between RES and CON (P=0.17) (Table 5.6.).

Table 5.6. Markers of calcium metabolism expressed as concentrations, percentage change from BASE and AUC in CON and RES trials. Mean values of D1 and D3 were used as BASE prior to each experimental condition.

	CON		RES	
	BASE	**D9**	**BASE**	**D9**
PTH				
pg·mL^{-1}	2.80 (1.14)	3.0 (1.16)	2.93 (1.00)	2.84 (0.67)
%BASE change		13.5 (34.7)		1.3 (26.0)
AUC (%BASE x d)[1]		65.9 (121.9)		-3.2 (97.4)
ACa				
mmol·L^{-1}	2.39 (0.06)	2.33 (0.05)	2.35 (0.05)	2.32 (0.06)
%BASE change		-2.2 (2.3)		-1.6 (2.7)
AUC (%BASE x d)[1]		-7.87 (8.27)		-1.00 (8.09)
Mg				
mmol·L^{-1}	0.84(0.05)	0.84(0.06)	0.84 (0.05)	0.85 (0.05)
%BASE change		1.3 (3.2)		1.6 (3.3)
AUC (%BASE x d)[1]		6.0 (19.7)		-8.2 (63.8)
PO_4				
mmol·L^{-1}	1.19 (0.12)	1.16 (0.17)	1.25 (0.16)	1.17 (0.14)
%BASE change		-2.4 (14.8)		-5.5 (10.7)
AUC (%BASE x d)[1]		14.5 (73.8)		-23.1(33.1)

Values are expressed as means (1SD).
[1]AUC calculated for each experimental condition from BASE to D9.
PTH: Parathyroid hormone; Mg: Magnesium; ACa: Albumin adjusted Calcium; PO_4: Phosphate; D: Day; BASE: Baseline; AUC: Area under the curve; CON, Controlled trial; RES, Restricted trial.

5.3.8. Regulatory hormones

5.3.8.1. Sclerostin

There was no significant main effect of time (P=0.10), condition (P=0.31) or any condition x time interaction effect (P=0.06) for mean sclerostin levels. Sclerostin AUC was not significantly different between CON and RES (P=0.31) (Table 5.7.).

5.3.8.2. IGF-1

There was a significant main effect of time (P<0.001) for mean IGF-1 concentrations, with significant decreases between BASE-D7, BASE-D9, D5-D7, D5-D9 and D7-D9 (all P values <0.005). A significant condition x time interaction effect (P=0.017) was shown, but there was no main effect of condition (P=0.45). At D9 IGF-1 concentrations were significantly reduced by 23.5% from BASE and by 20.3% from D5 in RES (P<0.001), but not in response to CON. IGF1 AUC was not significantly different between CON and RES (P=0.18) (Table 5.7.).

5.3.8.3. T_3

There was a significant main effect of time for T_3 concentrations (P=0.005), with mean concentrations being lower at D9 compared to BASE. No significant main effect of condition (P=0.30) or any condition x time interaction (P=0.17) was shown. T_3 AUC in RES was not significantly different from AUC in CON (P=0.09) (Table 5.7.).

5.3.8.4. Insulin

No significant main effects of time (P=0.09), condition (P=0.53) or any condition x time interaction effect (P=0.09) was shown for insulin concentrations. Insulin AUC after RES was not significantly different from AUC after CON (P=0.40) (Table 5.7.)

5.3.8.5. Leptin

Leptin was undetectable in 6 men; therefore, the analysis was performed in the remaining 5 men. There was a significant main effect of time (P=0.037) for leptin with concentrations at D5 being lower than at concentrations at D9. No significant main effect of condition (P=0.60) or any condition x time interaction effect (P=0.36) was shown. Leptin AUC after RES was not significantly different from AUC after CON (P=0.07) (Table 5.7.).

5.3.8.6. GLP-2

There was no significant main effect of time (P=0.10), condition (P=0.16) or any condition x time interaction effect (P=0.45) for GLP-2 concentrations. GLP-2 AUC was not significantly different between RES and CON (P=0.51) (Table 5.7.).

5.3.9. Individual analysis

The responders for β-CTX (increase) and P1NP (decrease) to RES were 3 and 6 out of 11 men respectively. Three out of 11 participants were responders to RES for both β-CTX (increase) and P1NP (decrease). In total, 6 out 11 men had altered bone turnover resulting from changes in bone resorption (increase), bone formation (decrease) or both (Table 5.8.).

Table 5.7. Regulatory hormones expressed as concentrations, percentage change from BASE and AUC in CON and RES trials (n=11). Mean values of D1 and D3 were used as BASE prior to each experimental condition

	CON		RES	
	BASE	**D9**	**BASE**	**D9**
Sclerostin				
ng·mL^{-1}	0.61 (0.15)	0.60 (0.18)	0.60 (0.14)	0.67 (0.16)
%BASE change		-1.9 (16.3)		10.4 (12.5)
AUC (%BASE x d)[1]		10.0 (46.8)		28.4 (41.1)
IGF-1				
mmol·L^{-1}	176.6 (57.6)	172.4 (72.3)	184.7 (55.6)	140.7 (42.9)*
%BASE change		-3.5 (20.3)		-23.5 (10.1)
AUC (%BASE x d)[1]		9.4 (96.9)		-54.0 (48.1)
T_3				
mmol·L^{-1}	1.75 (0.24)	1.66 (0.22)	1.80 (0.34)	1.56 (0.24)
%BASE change		-4.5 (8.7)		-12.9 (9.4)
AUC (%BASE x d)[1]		-6.4 (41.0)		-41.4 (47.6)
Leptin				
ng·mL^{-1}	2.62 (1.40)	2.54 (0.84)	3.89 (1.50)	1.93 (0.79)
%BASE change		11.2 (44.4)		-45.0 (26.8)
AUC (%BASE x d) [1]		158.9 (219.7)		-26.7 (331.0)
Insulin				
pmol·L^{-1}	33.0 (14.1)	32.4 (16.6)	35.1 (9.9)	18.6 (4.9)
%BASE change		8.7 (57.4)		-43.5 (22.2)
AUC (%BASE x d)[1]		23.0 (241.3)		-40.5(239.5)
GLP-2				
ng·mL^{-1}	15.0 (12.5)	13.9 (11.3)	14.1 (12.5)	10.4 (4.5)
%BASE change		-6.4 (13.6)		-8.8 (25.1)
AUC (%BASE x d)[1]		-27.0 (60.2)		-6.3 (50.3)

Values are expressed as means (1SD).
[1]AUC calculated for each experimental condition from BASE to D9.
[2] Analysis performed in 5 participants - leptin not detected in 6 participants.
*denotes a significant difference from BASE (P<0.05).
T_3: Triiodothyronine; IGF-1: Insulin-like growth factor 1; GLP-2: Glucagon-like peptide 2; D: Day; BASE: Baseline; AUC: Area under the curve; CON, Controlled trial; RES, Restricted trial.

Table 5.8. Number of responders (out of total number of participants, n=11) for β-CTX, P1NP and bone turnover. This analysis was based on data expressed as %BASE for each participant.

	***β*-CTX**	**P1NP**	**Bone turnover[1]**	**Bone turnover[2]**
RES	3/11	6/11	6/11	3/11

[1]altered bone turnover due to increase β-CTX, decreased P1NP or both.
[2]altered bone turnover due to a simultaneous increase in *β*-CTX and decrease in P1NP.
β-CTX: C-terminal cross-linked telopeptide of type I collagen; P1NP: Amino-terminal pro-peptide of type 1 procollagen; BT ratio; RES: Restricted.

5.4. Discussion

There are two main findings arising from the present study. Firstly, low EA at 15 kcal·kgLBM^{-1}·d^{-1} over 5 days had no significant effects on either bone formation (P1NP) or bone resorption (β-CTX) in men. Secondly, only IGF-1 was significantly reduced following short-term low EA, whereas all other regulatory hormones or markers of calcium metabolism were not affected by short-term low EA. These findings suggest that men are not sensitive to this level of reduced EA.

No previous, controlled, experimental study has examined the effects of low EA on bone turnover in men. In our investigation, bone formation was not significantly affected by reduced EA, despite the fact that P1NP decreased by 14%, which is similar to the 15% reduction in P1NP shown in male runners (Zanker & Swaine, 2000). The magnitude of the mean difference in P1NP was caused by some individuals who experienced a reduction in P1NP levels, compared with some others, whose P1NP concentrations did not change. This might explain the lack of a significant mean P1NP response in men in the present study, which is different from the more consistent response of P1NP to energy restriction shown by Zanker and Swaine (2000). Bone resorption remained unchanged in the present study, with this response being similar to those reported for markers of bone resorption in the earlier study by Zanker & Swaine (2000).

A negative association between bone turnover and low EA, similar to that established in women in the previous study (Chapter 4) and by Ihle & Loucks (2004) may become evident over more prolonged periods of exposure to the given level of EA in men. Studies of longer duration (8-12 weeks) in men have shown suppression of bone formation with or without changes in bone resorption in response to multi-stressor military training (exercise, dietary restriction and/or sleep deprivation) (Lutz et al., 2012; Hughes et al., 2014), although the contribution of each of these stressors to the observed bone turnover changes remain unknown. Altered BTM concentrations in favour of bone resorption have also been reported in male athletes participating in weight sensitive sports including runners (Hetland et al., 1993), jockeys (Wardon & Lynch, 2010; Dolan et al., 2012) and cyclists (Olmedillas et al., 2012), who may have been exposed to long-term energy restriction practices. The authors of these studies have not, however, provided any calculations of mean EA of these athletes. This limitation together with the cross-sectional design of these investigations does not allow the extrapolation of a cause-effect relationship between low EA and bone metabolism.

Another explanation may be that a more severe level of reduced EA is needed to elicit a consistent EA-associated change in BTM in men. This is supported by observational studies of low EA and bone health, demonstrating lower prevalence rates of the Triad symptoms (Tenforde et al., 2016) and stress fracture injury (Wentz et al., 2011; Armostrong et al., 2004; Bennell et al., 1996a) in men. Studies in male athletes suggest that low BMD is a finding generally confined to those partaking in weight category sports and experiencing multiple risk factors reflective of low EA such as low BMI, repeated bouts of rapid weight loss and/or disordered eating (Tenforde et al., 2016; Mountjoy et al., 2014; 2015).

The BTM response to reduced EA was characterised by high inter-individual variability, which suggested that some men were responders to low EA, while others remained unaffected. As such, it might be premature to suggest that low EA does not influence bone metabolism in all men. Importantly, the 3 men who responded to RES with increases in bone resorption (responders for β-CTX) also had reduced bone formation (responders for P1NP). These results may indicate an overall imbalance in bone metabolism with alterations favouring resorption. Additionally, 3 male participants responded with reduced bone formation (P1NP levels) only. The more consistent responses for P1NP are in agreement with previous studies showing that bone formation may be more sensitive than bone resorption to alterations in energy status (Ihle & Loucks, 2004). Although the mean BTM responses in response to low EA do not support the RED-S model in men (Mountjoy et al., 2014; 2015), this analysis of individual responses suggests some men experience negative bone consequences when exposed to low EA.

Our study population was representative of physically active individuals and strict inclusion and exclusion criteria were applied to eliminate confounding factors including age (Zanker & Swaine, 2000), training status (Scott et al., 2010) and body composition (Nguyen et al., 1998), making it unlikely that the variability shown in men was a result of these factors. Some of the observed inter-individual variability might be accounted for by genetic differences, which have previously been associated with bone health (Ralston & Uitterlinden, 2010) and injury (Varley et al., 2014), although, such speculations cannot be confirmed from the present study. In future research, it is necessary to understand what accounts for the observed variability and how this information can be used to develop strategies to prevent impairments in bone turnover in the affected individuals.

We also characterised the responses of energy regulatory hormones and markers of calcium metabolism in order to investigate, if these were related to BTM responses. A reduction in IGF-1 levels was shown after low EA, but no other change in any regulatory hormones or markers of calcium metabolism was seen. The significant reduction in IGF-1 concentrations in men in this study is similar to those reported in other laboratory (Zanker & Swaine, 2000) and field (Alemany et al., 2008; Hoyt & Friedl, 2006) studies involving energy restriction. The anabolic effects of IGF-1 on bone involve the stimulation of osteoblast differentiation, expression of type I collagen (Yakar & Rosen., 2003) and suppression of transcriptional factors that contribute to collagen breakdown (Canalis et al., 1995). Such effects were not supported by the BTM responses, suggesting that the suppression of IGF-1 was not sufficient to suppress bone turnover within the 5-day timeframe.

T_3 levels were not different between CON and RES in the present study, which is in agreement with a recent short-term study in men at the same level of low EA (15 kcal·kgLBM^{-1}·d^{-1}) and the same EEE contribution (15 kcal·kgLBM^{-1}·d^{-1}) (Koehler et al., 2016), but also with other short-term exercise-dietary restriction experiments (Hagobian et al., 2009). Exercise has been shown to upregulate T_3 production in adult male mice (Katzeff et al., 1988) and to maintain T_4 secretion and T_3 production in female Sprague-Dawley rats during exercise-induced energy deficit (Katzeff et al., 1991). In line with these exercise effects on thyroid hormones, which may mask T_3 responses in animal models, lower T_3 levels were not reduced in soccer players who followed low EA at some point during a competitive season (Reed et al., 2013). Leptin did not change in response to low EA. These results contrast those from a recent study showing decreased leptin using the same level of low EA (Koehler et al., 2016), but agree with other short-term interventions involving exercise training in overweight men (Kyriazis et al., 2007; Hagobian et al., 2009). Although leptin levels did not change, the mean magnitude of the response (-45%) was similar to that (-56%) reported by Koehler et al. (2016). A reduction in leptin levels by 36% was shown in non-exercising men when their DEI was restricted to 840 kcal·day^{-1} for 7 days (Dubuc et al., 1998). The authors did not provide quantification of EA; but based upon reported body composition and DEI data it could be estimated that EA was similar to our study (~14 kcal·kgFFM^{-1}·d^{-1}). The reductions (-43%) shown in insulin levels did not reach significance in our study. A significant 35% reduction in insulin levels was shown in exercising men at 15 kcal·kgLBM^{-1}·d^{-1} (Koehler et al., 2016), but not in overweight men after a 4-day exercise training and energy restriction programme (Hagobian et al., 2009).

We did not reveal any effects of low EA on PTH, ACa, Mg and PO_4; supporting the absence of significant BTM results in the present study. Calicum is the most abundant mineral of bone tissue playing a vital structural role. Calcium has also a plethora of metabolic functions including nerve conduction, muscle contraction, cell adhesion and blood coagulation (Miller et al., 2001). The importance of these metabolic roles necessitates its tight homeostatic control. A reduction in circulating calcium levels activates the release of PTH, which in turn, stimulates calcium resorption from bone in an effort to restore serum calcium levels (Adam & Hewison, 2010). Participants were provided with a daily multi-mineral supplement during RES; therefore, these results support the theory that we were successful in controlling overall micronutrient restriction and its possible effect on bone (Shapses & Sukumar, 2012).

We did not measure concentrations of reproductive hormones, and are, therefore, unable to conclude whether or not alterations of these factors have contributed to the observed bone-related outcomes. Both testosterone and oestrogen have been implicated in bone metabolism and health in men (Manolagas et al., 2013; Vanderschueren et al., 2014). Low BMD may be unrelated to testosterone concentrations in male athletes (Hetland et al., 1993; MacDougall et al., 1992; Maimoun et al., 2003), but an important role of oestrogen for the maintenance of the male skeleton is emerging (Vanderschueren et al., 2014). This was elegantly illustrated in a pilot study conducted by Ackerman et al. (2012b) where oestrogen levels were a stronger predictor of BMD than free or total testosterone in male collegiate runners and wrestlers, who may often experience periods of low EA. Current evidence to date is limited and future studies could provide more information about the relationship between sex steroids and low BMD in physically active men under low EA.

To conclude, this study has shown that low EA at 15 $kcal \cdot kgLBM^{-1} \cdot d^{-1}$ did not affect either bone formation or resorption in physically active men. IGF-1 was significantly reduced following low EA, but there was no effect on other regulatory hormones including T_3, insulin and leptin or on markers of calcium metabolism. Individual differences in susceptibility and responsiveness of BTMs to low EA were, however, shown, which might indicate that some, but not all, men are affected by low EA. Collectively, these results suggest that, overall, physically active men are resistant to acute reductions in low EA; but it would be premature to suggest that men are not affected by low EA. Future research should focus on investigating whether low EA at more severely restricted levels or for longer duration has different effects on bone metabolsim.

Chapter 6. Bone metabolic response to short-term low energy availability in physically active men and women: a sex comparison

6.1. Introduction

Most of the current studies on the effects of low EA on bone metabolism have been conducted in women. Ihle and Loucks (2004) explored the effects of low EA at 30, 20 and 10 kcal·kgLBM^{-1}·d^{-1} on bone turnover in sedentary women. We extended the results of this study in physically active females showing that low EA at 15 kcal·kgLBM^{-1}·d^{-1} elicited a reduction in bone formation as assessed by P1NP and a concurrent increase in bone resorption as indicated by changes in β-CTX (Chapter 4). Furthermore, in a group of physically active men, we were the first to demonstrate that overall, EA at 15 kcal·kgLBM^{-1}·d^{-1} had no effect on either bone formation or bone resorption (Chapter 5). These findings suggest that low EA may have a differential impact on bone metabolism in physically active women and men. In addition to our analyses within women and within men, our study design, prescription of the same level of low EA expressed relative to LBM, means that direct comparisons between sexes can be made.

Links between EA and bone health have been described in women under the Female Athlete Triad (Nattiv et al., 2007; De Souza et al., 2014a). Despite these interrelationships being more commonly seen in females (De Souza et al., 2014a); the concept of energy deficiency is not confined to physically active women, as suggested by more recent reports (Mountjoy et al., 2014; 2015; Tenforde et al., 2016). The recently introduced RED-S models highlight that all athletes, not just women may be affected by reduced EA (Mountjoy et al., 2014; 2015). The RED-S models have caused some controversy within the scientific community. Its supporters argue that RED-S models increase awareness of the effects of energy deficiency in all athletes that may be at risk (Mountjoy et al., 2015); whilst others argue that there is not enough information available in men compared to the well-established evidence-based Female Athlete Triad (De Souza et al., 2014a). Further studies in men and direct sex comparisons are needed in the area to provide more evidence and increase understanding of the effects of low EA on bone metabolism and health in men independently, but also relative to women.

Alterations in regulatory hormones in response to energy deficiency that may be related to alterations in bone metabolism have been identified (Walsh and Henriksen, 2010; Ihle & Loucks, 2004; Zanker & Zwaine, 2000), although sex-related responses have not been adequately investigated. Insulin and leptin have been shown to differ between men and women in response to changes in energy status in some (Hagobian et al., 2009; Dubuc et al., 1998; Hickey et al., 1997; Mittendorfer et al., 2001), but not all previous studies (Prouteau et al., 2006; Hagobian et al., 2013). Low EA may affect markers of reproductive function that

in turn, may also mediate bone responses (De Souza et al., 2008; Misra & Klibanski, 2011). Energy deficiency disrupts reproductive function in females (De Souza et al., 2007; Williams et al., 2015; Loucks & Thuma, 2003). Women with anorexia nervosa and female athletes under severe energy restriction typically present with amenorrhea (Miller, 2011; De Souza & Williams, 2005; 2010), whilst moderate levels of energy restriction also cause subclinical reproductive disorders in women (Williams et al., 2015; De Souza et al., 2010). The effects of energy deficiency on reproductive function in men are less consistent; with some studies demonstrating lower levels of reproductive hormones in male athletes participating in weight sensitive sports (Hackney et al., 1998; De Souza et al., 1994) and some others showing no effect (Lucia et al., 1996). Understanding sex-differences in these factors may improve our knowledge of the potential for different BTM responses in men and women and sex-dependent susceptibility for the development of bone disorders.

In this Chapter the data from Study 1 (women-presented in Chapter 4) and Study 2 (men-presented in Chapter 5) were combined to provide a direct sex comparison of BTM responses of men and women exposed to low EA at 15 $kcal \cdot kgLBM^{-1} \cdot d^{-1}$. A secondary goal was to explore potential sex-related differences in regulatory hormones and markers of calcium metabolism that may explain bone turnover responses.

6.2. Methods

6.2.1. Participants

In this analysis, data from twenty-two participants, eleven men and eleven eumenorrheic women (Table 6.2.) were included. The inclusion and exclusion criteria have been described in sections 4.2.1. for women and 5.2.1. for men.

6.2.2. Experimental design

Detailed description of the experimental design has been provided in section 4.2.2.

6.2.3. Experimental procedures

Detailed description has been provided for preliminary assessment in section 4.2.3.1., experimental period in section 4.2.3.2., lifestyle EE in section 4.2.3.2.1., habitual DEI in section 4.2.3.2.2. and 5.2.3., experimental diets in section 4.2.3.2.3., EEE in section 4.2.3.2.4 and compliance to experimental condition in section 4.2.3.2.5.

6.2.4. Storage and analyses of blood samples

Blood samples were collected and analysed as previously described in 4.2.4.

6.2.5. Biochemical analysis

Detailed description on biochemical analysis has already been provided in section 3.13.

6.2.6. Statistical analysis

All data were checked for normality according to the Shapiro-Wilk test and were log-transformed when not normally distributed before ANOVAs. Participant characteristics between men and women were compared using independent t-tests for normally distributed data or Wilcoxon-rank sum tests for non-normally distributed data. Similarly, baseline biochemistry markers and markers of energy status prior to each experimental condition were averaged and compared using independent t-tests or Wilcoxon-rank sum tests for non-normally distributed data. A three-way mixed model repeated measures ANOVA with sex (men, women) as a between subject factor and condition (CON, RES) and time (BASE, D5, D7 and D9) as within subject factors was used to assess group changes in BTM, regulatory hormones and markers of calcium metabolism. A two-way repeated measure ANOVA was used to determine differences over condition (CON and RES) between sexes for BTM, energy regulatory hormones and markers of calcium metabolism expressed as AUC.

Tukey's tests were used as *post hoc* analyses when a significant interaction effect was shown. Data are presented as mean (1SD) 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). For the individual analysis, criteria for responders and non-responders to RES for β-CTX and P1NP have been established in section 4.2.6.

6.3. Results

6.3.1. Participant characteristics

Demographic, body composition and training characteristics have been previously presented independently for women (please see section 4.3.1.) and men (please see section 5.3.1.) and sex differences are summarised in Table 6.1. Men were taller, heavier and had a greater BMI, LBM, FFM, BMD and VO_2 peak ($ml \cdot kg^{-1} \cdot min^{-1}$), but a lower body fat percentage than women ($P<0.05$). There were no differences in age, physical activity levels and VO_{2max} expressed relative to LBM ($P>0.05$). Men had a significantly higher absolute habitual DEI and estimated 24-h EE than women ($P<0.05$), but there were no significant differences in lifestyle EE between sexes ($P>0.05$) (Table 6.2.).

Table 6.1. Participant characteristics.

	Women (n=11)	**Men (n=11)**	**p-value**
Demographics			
Age (y)	26 (5)	26 (5)	0.85
Height (m)	1.66 (0.05)	1.78 (0.07)	<0.001*
Body mass (kg)	59.7 (6.7)	73.1 (8.0)	<0.001*
BMI ($kg \cdot m^{-2}$)	21.5 (1.5)	23.0 (1.6)	0.040*
Body composition			
Body fat (%)	27.1 (6.2)	18.3 (3.4)	0.001*
LBM (kg)	41.5 (4.9)	57.2 (7.3)	<0.001*
FFM (kg)	44.0 (5.1)	60.3 (7.6)	<0.001*
BMD ($g \cdot cm^{-2}$)	1.14 (0.11)	1.25 (0.08)	0.012*
Training characteristics			
VO_2 peak ($ml \cdot kg^{-1} \cdot min^{-1}$)	47.9 (5.5)	54.2 (5.3)	0.012*
VO_2 peak ($ml \cdot kg\ LBM^{-1} \cdot min^{-1}$)	68.7 (4.0)	69.3(5.9)	0.76
Physical activity ($MET\text{-}min \cdot wk^{-1}$)	3928 (1651)	3443 (1006)	0.42
Dietary and EE characteristics			
Habitual DEI ($kcal \cdot d^{-1}$)[1]	2143 (361)	2682 (265)	0.001*
Lifestyle EE ($kcal \cdot d^{-1}$)[1]	402 (227)	455 (136)	0.51
24-hour EE ($kcal \cdot d^{-1}$)[1]	1943 (302)	2398 (185)	<0.001

Values are presented as means (1SD).
[1] Mean values of D1-D3 prior to both experimental conditions.
*denotes a significant difference between men and women ($P<0.05$).
BMI: Body Mass Index; LBM: Lean Body Mass; FFM: Fat Free Mass; BMD: Bone mineral density: VO_{2max}: Maximum oxygen uptake; DEI: Dietary Energy Intake; EE: Energy expenditure; LBM: Lean Body Mass; MET: Metabolic equivalents.

6.3.2. Baseline biochemistry

Baseline concentrations of BTMs, markers of calcium metabolism and regulatory hormones have been previously presented for women (please see section 4.3.2.) and men (please see section 5.3.2.). Sex differences for these markers are summarised in Table 6.2. Women had significantly higher PTH levels, but lower ACa and sclerostin levels compared to men at BASE. There were no other differences in any BTM or any other markers of calcium metabolism or regulatory hormones (Table 6.2.).

Table 6.2. Concentrations of BTMs, markers of calcium metabolism and regulatory hormones at BASE in men and women. Mean values of D1 and D3 prior to both experimental conditions were used as BASE.

	Women (n=11)	**Men (n=11)**	**P-value**
BTMs			
***β*-CTX (µg·L^{-1})**	0.45 (0.12)	0.49 (0.16)	0.53
P1NP (µg·L^{-1})	70.9 (13.6)	74.3 (29.4)	0.73
BT ratio	1.63 (0.40)	1.54 (0.40)	0.60
Markers of calcium metabolism			
PTH (pg·mL^{-1})	3.8 (0.6)	2.9 (1.0)	0.012*
ACa (mmol·L^{-1})	2.32 (0.04)	2.37 (0.05)	0.013*
Mg (mmol·L^{-1})	0.83 (0.04)	0.83 (0.05)	0.92
PO_4 (mmol·L^{-1})	1.23 (0.14)	1.22 (0.11)	0.90
Regulatory hormones			
Sclerostin (ng·mL^{-1})	0.46 (0.12)	0.61 (0.14)	0.015*
IGF-1 (mmol·L^{-1})	229.1 (54.8)	180.7 (52.4)	0.37
T_3 (mmol·L^{-1})	1.66 (0.20)	1.78 (0.28)	0.22
Leptin (ng·mL^{-1})[1]	9.7 (13.5)	2.9 (1.6)	0.25
Insulin (pmol·L^{-1})	40.2 (25.8)	34.0 (10.7)	0.35
GLP-2 (ng·mL^{-1})	10.0 (6.7)	14.5 (12.4)	0.29

Values are presented as means (1SD).
[1]Analysis performed in 6 men and 11 women.
*denotes a significant difference between men and women ($P<0.05$).
β-CTX: C-terminal cross-linked telopeptide of type I collagen; P1NP: Amino-terminal pro-peptides of type 1 procollagen; BT ratio: Bone turnover ratio; BTM: Bone turnover markers; 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; D: Day; BASE: Baseline.

6.3.3. BTMs

6.3.3.1. β-CTX

There was no significant condition x time x sex interaction (P=0.25), nor were there any condition x sex (P=0.12) or condition x time (P=0.44) interaction effects for β-CTX concentrations. There was a significant main effect of time (P=0.002), β-CTX concentrations at D7 and D9 were greater than BASE, but no significant main effect of condition (P=0.91) or sex (P=0.67). The between sexes comparison showed a significant condition x sex interaction effect for β-CTX AUC (P=0.03) only, although no pairwise *post hoc* comparisons reached statistical significance (Figure 6.1., Figure 6.2., Table 6.3.).

6.3.3.2. P1NP

There was no significant condition x time x sex or condition x sex interactions for P1NP concentrations, although there was a condition x time interaction (P=0.01). P1NP concentrations decreased between BASE-D5 (P=0.04), BASE-D7 (P<0.001) and BASE-D9 (P<0.001) in RES, but not in CON. P1NP concentrations at D7 (P=0.02) and D9 (P=0.002) in RES were also lower than in CON. A significant main effect of time (P<0.001) showed that P1NP concentrations reduced progressively from D5 to D9 compared to BASE (P<0.01). There were no significant main effects of condition (P=0.13) or sex (P=0.91). A significant main effect of condition was evident for P1NP AUC (RES<CON, P=0.008). There was no significant main effect of sex (P=0.48) or any condition x sex interaction effect (P=0.37) for P1NP AUCs (Figure 6.1., Figure 6.2., Table 6.3.).

6.3.3.3. BT ratio

There was no significant condition x time x sex (P=0.35) or condition x sex (P=0.6) interaction effects, although a significant condition x time interaction (P=0.01) was shown. The BT ratio decreased progressively from BASE to D9 in RES (BASE-D5, P=0.01; BASE-D7, P<0.001; BASE-D9, P<0.001; D5-D9, P=0.02), but not in CON. BT ratios at D7 (P=0.02) and D9 (P=0.002) in RES were also lower than those at the same time points in CON. There was a significant main effect of time for BT ratio (BASE-D5; BASE-D7; BASE-D9; P<0.05), but no significant main effect of condition (P=0.32) or sex (P=0.27). There was a significant main effect of condition (RES<CON; P=0.01), but no significant main effect of sex (P=0.48) or any condition x sex interaction effect (P=0.37) shown for BT ratio AUC (Figure 6.1., Figure 6.2., Table 6.3.).

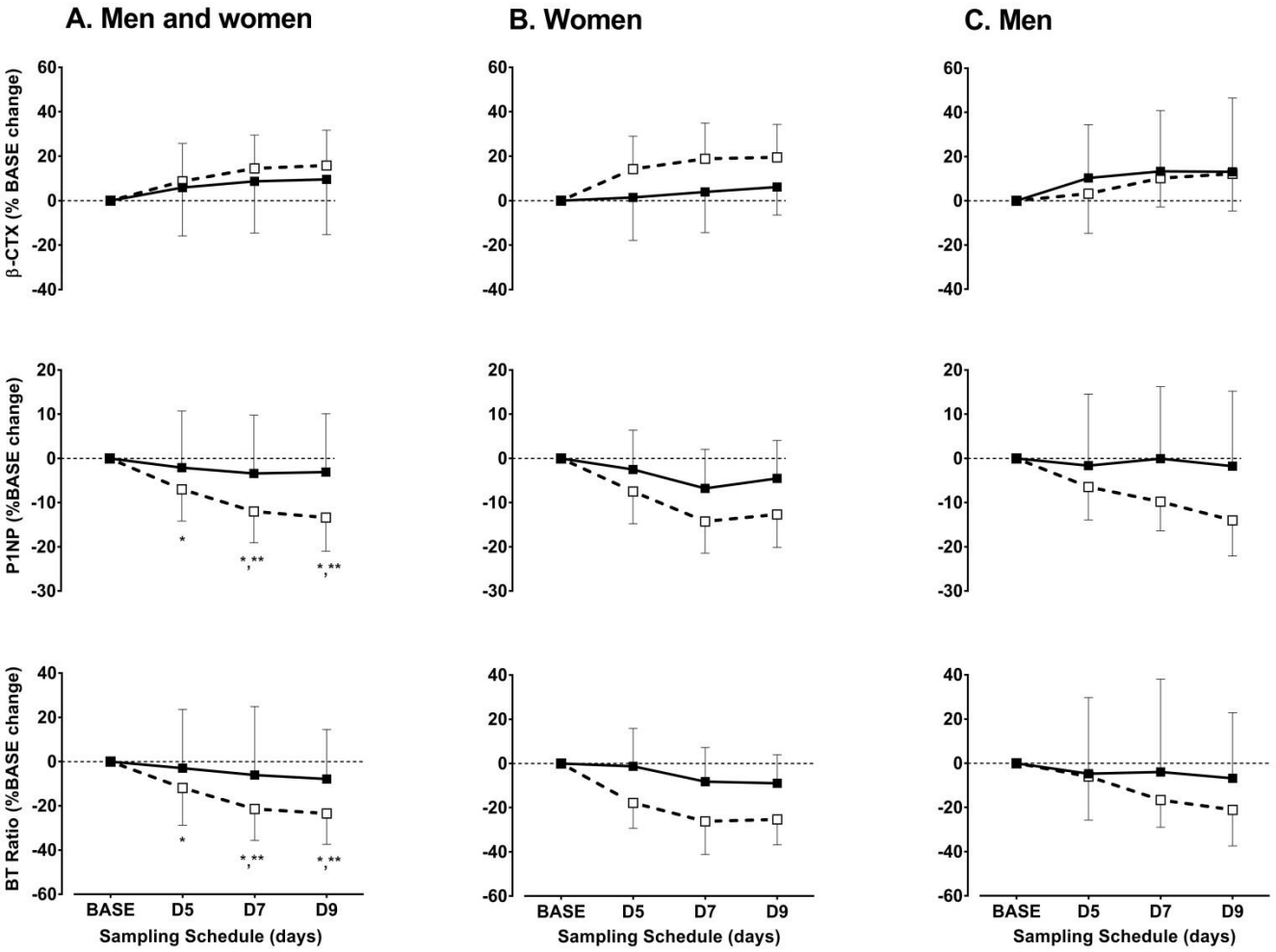


Figure 6.1. Percentage change from BASE for β-CTX, P1NP and BT ratio on D5-D9 in CON (black squares) and RES (white squares) trials in (A) men and women, (B) women and (C) men. Mean values of D1 and D3 were used as BASE prior to each experimental condition. Values are presented as means (1SD). * denotes a significant difference from BASE ($P<0.05$).). ** denotes a significant difference from CON at the same timepoint ($P<0.05$). β-CTX: C-terminal cross-linked telopeptide of type I collagen; P1NP: Amino-terminal pro-peptide of type 1 procollagen; BT ratio; D: Day; BASE: Baseline.

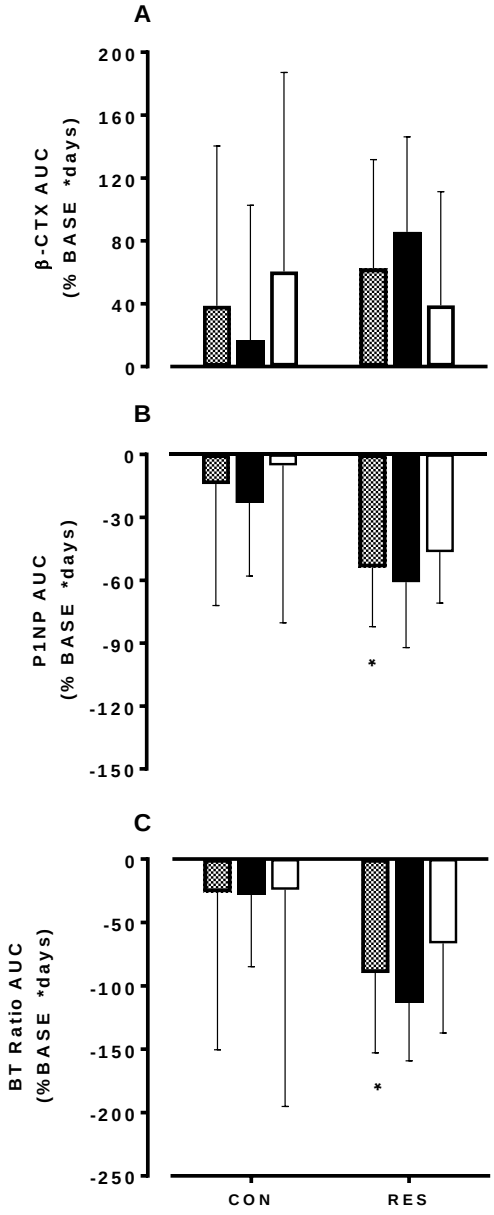


Figure 6.2. AUC for *β*-CTX (A), P1NP (B) and BT ratio (C) in women and men together (black and white pattern), women (black bars) and men (white bars). Values are presented as means (1SD). * denotes a significant difference between CON and RES ($P<0.05$). β-CTX: C-terminal telopeptides of type I collagen; P1NP: Amino-terminal pro-peptide of type 1 procollagen; BT ratio: Bone turnover ratio; BASE: Baseline; AUC: Area under the curve CON: Controlled; RES: Restricted; BASE: Baseline.

Table 6.3. BTMs expressed as concentrations, percentage change from BASE and AUC in CON and RES trials in men and women combined data (total), women and men. Mean values of D1 and D3 were used as BASE prior to each experimental condition.

	Total (n=22)				**Women (n=11)**				**Men (n=11)**			
	CON		**RES**		**CON**		**RES**		**CON**		**RES**	
	BASE	**D9**	**BASE**	**D9**	**BASE**	**D9**	**BASE**	**D9**	**BASE**	**D9**	**BASE**	**D9**
***β*-CTX**												
µg·L^{-1}	0.48	0.51	0.46	0.53	0.49	0.51	0.42	0.49	0.48	0.52	0.50	0.57
	(0.14)	(0.15)	(0.15)	(0.20)	(0.14)	(0.12)	(0.11)	(0.09)	(0.16)	(0.17)	(0.17)	(0.26)
%BASE change		9.6		15.8		6.1		19.5		13.1		12.2
		(24.9)		(15.9)		(12.6)		(14.8)		(33.3)		(16.8)
AUC		38.7		62.4		16.9		85.7		60.5		39.0
(%BASE x d) [1]		(101.7)		(69.3)		(68.1)		(60.5)		(126.7)		(72.3)
P1NP												
µg·L^{-1}	72.4	70.0	72.7	62.9	71.1	67.5	70.6	61.6	73.6	72.5	74.8	64.3
	(24.0)	(27.2)	(22.5)	(20.0)*,**	(15.0)	(13.7)	(15.1)	(14.0)	(31.4)	(36.7)	(28.7)	(25.3)
%BASE change		-3.1		-13.4		-4.5		-12.7		-1.8		-14.0
		(13.2)		(7.6)		(8.6)		(7.4)		(16.2)		(8.0)
AUC		-14.1		-53.8		-23.1		-60.9		-5.2		-46.6
(%BASE x d)[1]		(57.9)		(28.3)**		(34.9)		(31.2)		(75.1)		(24.3)
BT ratio												
-	1.55	1.39	1.62	1.22	1.54	1.39	1.73	1.27	1.57	1.39	1.52	1.16
	(0.44)	(0.40)	(0.41)	(0.30)*,**	(0.44)	(0.39)	(0.42)	(0.33)	(0.46)	(0.42)	(0.40)	(0.29)
%BASE change		-7.9		-23.5		-9.0		-25.3		-6.8		-21.1
		(22.4)		(13.9)		(12.9)		(11.4)		(29.7)		(16.3)
AUC		-26.1		-89.9		-28.0		-113.4		-24.2		-66.4
(%BASE x d) [1]		(124.4)		(63.0)**		(56.9)		(45.8)		(171.0)		(70.8)

Values are expressed as means (1SD). [1]AUC calculated for each experimental condition from BASE to D9. * denotes a significant difference from BASE in the same condition (P<0.05). **denotes a significant difference from CON at the same timepoint (P<0.05). *β*-CTX: C-terminal cross-linked telopeptide of type I collagen; P1NP: Amino-terminal pro-peptide of type 1 procollagen; BT ratio: Bone turnover ratio; BTM: Bone turnover marker; D: Day; BASE: Baseline; AUC: Area under the curve CON: Controlled trial; RES: Restricted trial.

6.3.4. Markers of calcium metabolism

6.3.4.1. PTH

There was no significant condition x time x sex interaction effect (P=0.22), nor were there any condition x sex (P=0.76) or condition x time (P=0.81) interaction effects for PTH concentrations. There was a significant main effect of time, indicating greater PTH concentrations at D7 compared to BASE (P=0.02) and a main effect of sex (P<0.001) showing higher concentrations in women than in men. For PTH AUC, no significant main effects of condition (P=0.39), sex (P=0.32) or any condition x sex interaction effect (P=0.65) was shown (Table 6.4.).

6.3.4.2. ACa

There was no significant condition x time x sex interaction effect (P=0.85), nor was there any condition x sex (P=0.24) or any condition x time (P=0.06) interaction effect for ACa levels. A significant time x sex interaction (P=0.01) was shown; in men ACa significantly decreased at D9 compared to BASE (P=0.035) and D5 (P=0.048). There was no significant main effect of time (P=0.053), condition (P=0.67) or sex (P=0.08). For ACa AUC, no significant main effect of condition (P=0.052), sex (P=0.067) or any condition x sex interaction (P=0.25) was shown (Table 6.4.).

6.3.4.3. Mg

There was no significant condition x time x sex interaction effect (P=0.37), nor were there any condition x sex (P=0.48) or condition x time (P=0.57) interaction effects for Mg levels. There were no significant main effects of time (P=0.75) condition (P=0.47) or sex (P=0.67) for Mg levesls. For Mg AUC, no significant main effect of sex (P=0.52), condition (P=0.75) or any condition x time interaction effect was shown (P=0.30) (Table 6.4.).

6.3.4.4. PO_4

There was a significant condition x time x sex interaction (P=0.02) for PO_4 concentrations only, but *post-hoc* analysis did not discover any significant differences. No significant main effect of time (P=0.23), condition (P=0.92) or sex (P=0.32) were shown for PO_4 levels. The between sexes comparison showed no significant main effect of sex (P=0.40), condition (P=0.28) or any condition x time interaction effect (P=0.11) for PO_4 AUC (Table 6.4.).

6.3.5. Regulatory hormones

6.3.5.1. Sclerostin

There was no significant condition x time x sex interaction effect (P=0.39), nor were there any condition x sex (P=0.55) or condition x time (P=0.10) interaction effects for sclerostin concentrations. There was a significant main effect of time (P=0.01); increases were shown at D7 compared to BASE. There was a main effect of sex (P=0.01) with higher levels shown in men compared to those in women, but no significant main effect of condition was shown (P=0.52) for sclerostin concentrations. For sclerostin AUC, no main effects of sex (P=0.55), condition (P=0.82), or any condition x sex interaction effect (P=0.14) was shown (Table 6.5).

6.3.5.2. IGF-1

In RES only, IGF-1 concentrations at D7 were reduced from BASE (P<0.001) and concentrations at D9 were lower than those at BASE (P<0.001) and D5 (P<0.001) (overall, a significant condition x time interaction effect, P=0.003). In men, IGF1 levels were lower at D7 compared to BASE and at D9 compared to BASE and D5 (P<0.05). In women, reductions of IGF-1 concentrations were shown between BASE-D7, BASE-D9, D5-D7 and D5-D9 (all P values <0.05) (overall, a significant time x sex interaction effect, P=0.017). IGF-1 concentrations were not different in any condition for any time point between men and women (condition x time x sex interaction effect, P=0.76). For pooled men and women data, there was no difference between condition for any time point (condition x sex interaction effect, P=0.69). For IGF-1 concentrations there was a significant main effect of time (P<0.001), overall, reductions were shown between BASE-D7, BASE-D9, D5-D7 and D5-D9 (P<0.001). There was no main effect of condition (P=0.43) or sex (P=0.15). The between sexes comparisons showed a main effect of sex (women<men, P=0.04) and condition (RES<CON, P=0.02), but no condition x sex interaction effect for IGF-1 AUC (Table 6.5).

6.3.5.3. T_3

There was no significant condition x time x sex (P=0.43) or condition x sex (P=0.84) interaction effect, although there was a significant condition x time interaction effect (P=0.003). T_3 at D7 was lower compared to BASE (P<0.001) and at D9 was decreased from D5 (P=0.001) and BASE (P<0.001) in RES only. T_3 concentrations at D9 in RES were also lower than T_3 concentrations at D9 in CON (P=0.001). A significant main effect of time (D7<BASE, D9<BASE; P<0.05) was shown for T_3 concentrations, but there was no main

effect of sex (P=0.29) or condition (P=0.06). A significant main effect of condition was shown for T_3 AUC (P=0.01), showing that T_3 responses were lower in RES than in CON (Table 6.5), but there was no significant main effect of sex (P=0.68) or condition x sex interaction effect (P=0.82).

6.3.5.4. Leptin

There was no significant condition x time x sex (P=0.96) or condition x sex (P=0.29) interaction effect. There was only a significant condition x time interaction (P=0.007); leptin concentrations decreased significantly from BASE at D5 (P=0.02), D7 (P<0.001) and D9 (P<0.001). Leptin at D9 in RES was lower from D9 in CON (P=0.04). There was a significant main effect of time (P<0.001), but no main effect of sex (P=0.28) or condition (P=0.83). Significant main effects of sex (Women<Men, P=0.02) and condition (RES<CON, P=0.003) were shown for leptin AUC (Table 6.5), but there was no significant condition x sex interaction effect (P=0.30).

6.3.5.5. Insulin

There was no condition x time x sex (P=0.32) or condition x sex (P=0.11) interaction effect for insulin concentrations, although there was a condition x time interaction effect (P<0.001). Insulin concentrations decreased significantly from BASE at D5 (P=0.007), D7 (P<0.001) and D9 (P<0.001). Insulin concentrations at D9 in RES were lower than the concentration at the respective time point in CON (P<0.001). Significant main effects of time (D7<BASE and D9<BASE; P=0.002) and condition (RES<CON; P=0.017) were shown for insulin concentrations, but there was no main effect of sex (P=0.94). For insulin AUC, there was a significant main effect of condition (RES<CON, P=0.02), but no main effect of sex (P=0.25) or any sex x condition interaction effect (P=0.28) (Table 6.5.).

6.3.5.6. GLP-2

There was no significant condition x time x sex interaction (P=0.46), nor were there any condition x sex (P=0.44) or condition x time (P=0.52) interaction effects for GLP-2 concentrations. No significant main effect of time (P=0.13), condition (P=0.08) or sex (P=0.29) were shown for GLP-2 levels. For GLP-2 AUC, there was no significant main effect of sex (P=0.53), condition (P=0.17) or any condition x time interaction (P=0.80) (Table 6.5.).

Table 6.4. Markers of calcium metabolism expressed as concentrations, percentage change from BASE and AUC in CON and RES trials in men and women combined data (total), women and men. Mean values of D1 and D3 were used as BASE prior to each experimental condition.

	Total (n=22)				**Women (n=11)**				**Men (n=11)**			
	CON		**RES**		**CON**		**RES**		**CON**		**RES**	
	BASE	**D9**	**BASE**	**D9**	**BASE**	**D9**	**BASE**	**D9**	**BASE**	**D9**	**BASE**	**D9**
PTH												
pg·mL^{-1}	3.4	3.6	3.3	3.5	4.0	4.2	3.7 (0.7)	4.3	2.80	3.0	2.9	2.8
	(1.1)	(1.2)	(0.9)	(1.1)	(0.7)	(1.0)		(1.0)	(1.2)	(1.2)	(1.00)	(0.7)
%BASE change		9.0		8.5		4.6		15.7		13.5		1.3
		(27.2)		(22.8)		(17.6)		(17.2)		(34.7)		(26.0)
AUC		57.9		36.4		49.9		76.1		65.9		-3.2
(%BASE x d)[1]		(93.9)		(97.0)		(59.4)		(82.6)		(121.9)		(97.4)
ACa												
mmol·L^{-1}	2.35	2.33	2.33	2.32	2.32	2.33	2.32	2.32	2.39	2.33	2.35	2.32
	(0.07)	0.06	(0.05)	0.05	(0.05)	(0.06)	(0.05)	(0.04)	(0.06)	(0.05)	(0.05)	(0.06)
%BASE change		-0.7		-0.8		0.8		0.0		-2.2		-1.6
		(2.5)		(2.3)		(1.8)		(1.6)		(2.3)		(2.7)
AUC		-4.4		-0.0		-0.9		1.0		-7.9		-1.00
(%BASE x d)[1]		(7.9)		(7.4)		(5.9)		(6.8)		(8.3)		(8.1)
Mg												
mmol·L^{-1}	0.84	0.84	0.83	0.85	0.84	0.84	0.83	0.85	0.83	0.84	0.83	0.85
	(0.05)	(0.05)	(0.05)	(0.05)	(0.05)	(0.04)	(0.04)	(0.05)	(0.05)	(0.06)	(0.05)	(0.05)
%BASE change		1.1		1.8		0.8		2.0		1.3		1.6
		(2.9)		(3.8)		(2.7)		(4.4)		(3.2)		(3.3)
AUC		4.3		1.0		2.5		10.1		6.0		-8.2
(%BASE x d)[1]		(18.3)		(47.3)		(17.6)		(21.3)		(19.7)		(63.8)

Markers of calcium metabolism (continue)	Total (n=22)				Women (n=11)				Men (n=11)			
	CON		RES		CON		RES		CON		RES	
	BASE	D9	BASE	D9	BASE	D9	BASE	D9	BASE	D9	BASE	D9
PO_4												
$mmol \cdot L^{-1}$	1.21	1.23	1.24	1.20	1.22	1.31	1.23	1.23	1.19	1.16	1.25	1.17
	(0.12)	(0.18)	(0.16)	(0.14)	(0.12)	(0.17)	(0.17)	(0.13)	(0.12)	(0.17)	(0.16)	(0.14)
%BASE change		2.4		-2.3		7.3		1.0		-2.4		-5.5
		(12.3)		(9.9)		(6.7)		(8.4)		(14.8)		(10.7)
AUC		15.7		-2.1		17.0		18.9		14.5		-23.1
(%BASE x d)[1]		(57.0)		(40.0)		(36.9)		(36.1)		(73.8)		(33.1)

Values are expressed as means (1SD).

[1]AUC calculated for each experimental condition from BASE to Day 9.

PTH: Parathyroid hormone; Mg: Magnesium; ACa: Albumin adjusted Calcium; PO_4: Phosphate; D: Day; BASE: Baseline; AUC: Area under the curve; CON, Controlled trial; RES, Restricted trial.

Table 6.6. Regulatory hormones expressed as concentrations, percentage change from BASE and AUC in CON and RES trials in men and women combined data (total), women and men. Mean values of D1 and D3 were used as BASE prior to each experimental condition.

	Total (n=22)				**Women (n=11)**				**Men (n=11)**			
	CON		**RES**		**CON**		**RES**		**CON**		**RES**	
	BASE	**D9**	**BASE**	**D9**	**BASE**	**D9**	**BASE**	**D9**	**BASE**	**D9**	**BAS**	**D9**
Sclerostin												
ng·mL^{-1}	0.53	0.54	0.53	0.58	0.45	0.47	0.46	0.49	0.61	0.60	0.60	0.67
	(0.16)	(0.16)	(0.14)	(0.17)	(0.13)	(0.12)	(0.11)	(0.13)	(0.15)	(0.18)	(0.14)	(0.16)
%BASE change		2.7		7.6		6.8		4.8		-1.9		10.4
		(17.0)		(10.8)		(17.3)		(8.5)		(16.3)		(12.5)
AUC		23.6		20.3		28.8		3.1		18.3		37.4
(%BASE x d)[1]		(68.4)		(44.7)		(81.6)		(27.8)		(55.6)		(52.6)
IGF-1												
mmol·L^{-1}	199.9	187.2	209.9	163.4	223.2	202.0	235.0	186.1	176.6	172.4	184.7	140.7
	(61.4)	(65.7)	(64.6)	(54.7)*	(58.4)	(57.8)	(65.5)	(57.5)*	(57.6)	(72.3)	(55.6)	(42.9)*
%BASE change		-6.1		-22.3		-8.8		-21.1		-3.5		-23.5
		(20.3)		(9.6)		(20.9)		(9.3)		(20.3)		(10.1)
AUC		-19.4		-72.5		-48.2		-90.9		9.4		-54.0
(%BASE x d)[1]		(92.7)		(50.2)**		(82.6)		(47.2)		(96.9)		(48.1)
T_3												
mmol·L^{-1}	1.70	1.66	1.73	1.52	1.65	1.66	1.66	1.47	1.75	1.66	1.80	1.56
	(0.24)	(0.21)	(0.28)	(0.21)*,**	(0.25)	(0.22)	(0.19)	(0.18)	(0.24)	(0.22)	(0.34)	(0.24)
%BASE change		-1.3		-11.8		1.9		-10.7		-4.5		-12.9
		(8.4)		(9.7)		(7.0)		(10.1)		(8.7)		(9.4)
AUC		-4.1		-36.4		-1.8		-31.5		-6.4		-41.4
(%BASE x d)[1]		(51.0)		(43.4)**		(61.3)		(40.5)		(41.0)		(47.6)

Energy regulatory hormones (continue)	Total (n=22)				Women (n=11)				Men (n=11)			
	CON		RES		CON		RES		CON		RES	
	BASE	D9	BASE	D9	BASE	D9	BASE	D9	BASE	D9	BASE	D9
Leptin												
pmol·L^{-1} [2]	6.5	4.8	8.9	3.5	8.3	5.9	11.1	4.2	2.6	2.5	3.9	1.9
	(9.4)	(9.4)	(13.6)	(4.3)*,**	(11.0)	(5.7)	(16.1)	(5.0)	(1.4)	(0.8)	(1.5)	(0.8)
%BASE change[2]		-2.1		-43.4		-8.8		-42.7		11.2		-45.0
		(39.6)		(34.3)		(34.9)		(38.5)		(44.4)		(26.8)
AUC		-31.7		-157.6		-118.3		-214.2		158.9		-26.7
(%BASE x d)[1,2]		(199.8)		(24.6)**		(119.0)		(113.0)		(219.7)		(331.1)
Insulin												
pmol·L$^{-1(1)}$	34.5	34.8	39.6	20.5	36.1	37.3	44.2	22.4	33.0	32.4	35.1	18.6
	(13.3)	(15.7)	(19.6)	(9.8)	(12.9)	(15.1)	(25.8)	(13.0)	(14.1)	(16.6)	(9.9)	(4.9)
%BASE change		13.9		-40.7		19.2		-37.9		8.7		-43.5
		(56.5)		(28.3)*,**		(57.9)		(34.2)		(57.4)		(22.2)
AUC		3.3		-110.5		-16.5		-180.5		23.0		-40.5
(%BASE x d)[1]		(214.7)		(200.2)**		(194.2)		(126.6)		(241.3)		(239.5)
GLP-2												
ng·mL^{-1}	12.8	11.7	11.7	10.1	10.5	9.5	9.4	9.8	15.0	13.9	14.1	10.4
	(10.4)	(8.8)	(9.7)	5.6	(7.7)	(4.9)	(5.6)	(6.8)	(12.5)	(11.3)	(12.5)	(4.5)
%BASE change		-5.2		-3.5		-4.0		1.8		-6.4		-8.8
		(15.5)		(22.0)		(17.8)		(18.2)		(13.6)		(25.1)
AUC		-23.6		-1.7		-20.2		9.6		-27.0		-6.3
(%BASE x d)[1]		(64.0)		(50.0)		(70.5)		(50.8)		(60.2)		(50.3)

Values are expressed as means (1SD). [1]AUC calculated for each experimental condition from BASE to Day 9. [2]Analysis performed in 5 men and 11 women.

*denotes a significant difference from BASE in the same condition (P<0.05). **denotes a significant difference for CON at the same point (P<0.05).

T_3: Triiodothyronine; IGF-1: Insulin-like growth factor 1; GLP-2: Glucagon-like peptide 2; D: Day; BASE: Baseline; AUC: Area under the curve; CON, Controlled trial; RES, Restricted trial.

6.3.7. Individual analysis

Three out of 11 men responded to RES with an increase in β-CTX concentrations and 6 out of 11 men responded to RES with a decrease in P1NP concentrations. Furthermore, 3 out of 11 male participants were responders to RES for both *β*-CTX (increase) and P1NP (decrease). In women, 7 out of 11 participants responded to RES with an increase in β-CTX concentrations and 6 out of 11 participants responded to RES with a decrease in P1NP concentrations. Four female participants were responders for *β*-CTX only (increase), 3 participants were responders for P1NP only (decrease) and 3 participants responded to RES for both. In total, 6 out of 11 men and 10 out 11 women had altered bone turnover resulting from changes in bone resorption (increase), bone formation (decrease) or both (Table 6.5.).

Table 6.5. Number of responders (out of total number of women, men and total) for *β*-CTX, P1NP in physically active women and men in RES. This analysis was based on data expressed as %BASE for each participant.

	***β*-CTX**	**P1NP**	**Bone turnover[1]**	**Bone turnover[2]**
Women (n=11)	7/11	6/11	10/11	3/11
Men (n=11)	3/11	6/11	6/11	3/11
Total (n=22)	10/22	12/22	16/22	6/22

[1]altered bone turnover due to increase *β*-CTX, decreased P1NP or both.
[2]altered bone turnover due to a simultaneous increase in *β*-CTX and decrease in P1NP.
β-CTX: C-terminal cross-linked telopeptide of type I collagen; P1NP: Amino-terminal pro-peptide of type 1 procollagen; BT ratio; RES: Restricted; BASE: Baseline.

6.4. Discussion

There were no significant differences in bone metabolism, regulatory hormones or markers calcium metabolism in response to low EA (achieved by diet and exercise) at 15 kcal·kgLBM^{-1}·d^{-1} between sexes. This sex comparison provides a different insight from the findings of our single sex studies, which indicated significant alterations in bone formation and resorption in response to low EA in women, but not in men. When combining the data from men and women, a significant reduction in bone formation was shown in response to low EA, without any concomitant effect on bone resorption, suggesting that bone formation may be affected first in conditions of energy deficiency and may result in an uncoupling of bone formation from bone resorption. The changes in BTMs in response to low EA were accompanied by reductions in key hormones, namely IGF-1, T_3, insulin and leptin.

In this study, a direct sex comparison has been made using the same research design and protocols [*i.e.*, prescription of the same level of low EA (relative to LBM) and the same contributions from dietary restriction and exercise] for both women and men. This comparison is novel in the area of bone metabolism and low EA, as no previous study has examined both sexes concurrently within the same analysis. No significant differences were shown in BTM responses following low EA in men and women. Interestingly, the magnitudes of the changes in BTMs were similar between sexes (Men - β-CTX: +12%, P1NP: -14%; BT ratio: -21%; Women - β-CTX: +19%, P1NP: -13%; BT ratio: -24%). These results differ from the results of the within group comparisons, which showed an increase in bone resorption and reductions in bone formation and the BT ratio in women (Chapter 4), but no effect in men (Chapter 5). Therefore, our findings in women support previous studies showing that low EA underpins bone health in women (De Souza et al., 2014a). However, our findings in men do not support the RED-S phenomenon when considered alone, but may suggest an analogous relationship with women when considering the similar BTM responses shown in this study between men and women to low EA.

Although there is no previous study on sex-related differences in BTM responses to short-term low EA, findings from cross-sectional studies on sex-differences in bone-related outcomes, that are mediated by changes in bone turnover, such as BMD and bone injuries are inconsistent. Studies in men and women exposed to multi-stressor environments including low EA have suggested that women may be more susceptible to the effects of low EA with higher incidence rates of stress fracture injury (Wentz et al., 2011; Armstrong et al.,

2004; Bennell et al., 1996a) and low BMD (Tenforde et al. 2016; De Souza et al., 2014a; Mountjoy et al., 2015). Other studies, however, showed no sex differences in BMD (Hind et al., 2006) or stress fracture incidence rates (Matheson et al., 1987; Iwamoto & Takeda, 2003). Based on these findings, firm conclusion regarding sex differences in bone related outcomes cannot be drawn. The results of these studies should be interpreted with caution, as male and females were not exposed to low EA only or to the same level of low EA. Furthermore, although these studies evaluated longer-term bone outcomes, the nature of these alterations (*i.e.,* low BMD resulting from reduced bone formation, elevated bone formation or both) has not been investigated.

In the present study, individual analysis was conducted in order to supplement conventional statistics. Studies typically emphasise mean responses; however, individuals may differ considerably in response to exercise and/or dietary restriction (King et al., 2008). Six out of 11 men and 10 out 11 women had 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 amongst our female participants. Inter-individual variability to energy deficiency has been reported in body composition changes (King et al., 2008), insulin sensitivity (King et al., 2012), and female reproductive function (Williams et al., 2015), which may be relevant to sex-specific bone turnover responses. Individual analysis may be valuable in identifying individuals or subgroups that are more susceptible to low EA and may benefit from securing optimal EA.

Since there were no sex differences in BTMs, by combining data in men and women, a significant reduction in P1NP by 13% from BASE, together with a significant 24% reduction in BT ratio in response to RES were shown, with these results suggesting an imbalance in bone turnover. These findings are in line with the findings of a previous, short-term experiment conducted in women suggesting that bone formation is altered first (bone resorption follows) in response to changes in energy status (Ihle & Loucks, 2004). In contrast, using an integrated sex approach (analysis of combined men and women data), Villareal et al. (2016) showed a marked increase in bone resorption (approximately 30% increase from BASE) as assessed by β-CTX and TRACP5b in 143 non-obese individuals (BMI: 25.1±1.7 $kg \cdot m^{-2}$) after six months and 1-year of 25% caloric restriction. For bone formation, there was a transient decrease (-6% form BASE) in BALP at 1-year follow-up, but P1NP remained unaltered (Villareal et al., 2016). The absence of mechanical loading

(energy restriction was achieved by food restriction only), the longer study duration and follow-up and the differences in study demographics do not allow direct comparison with the findings of the present study. However, the study by Villareal et al. (2016) also suggests an imbalance between bone formation and resorption, in favour of resorption. This is important given that altered bone turnover favouring bone resorption, if continued, can result in bone loss (Villareal et al., 2016), changes in bone architecture and bone injuries (Hernadez, 2008).

Alterations in a number of regulatory hormones have been shown to mediate some of the BTM responses (Zanker & Swaine, 2000; Ihle and Loucks, 2004; Villareal et al., 2016), although no previous experimental study has provided a direct sex comparison of regulatory hormones whilst assessing bone-related outcomes. Responses of regulatory hormones to low EA did not vary between sexes, supporting the absence of sex differences in BTM responses. The direction and magnitude of regulatory hormone responses were similar between men and women (Men: IGF-1: -24%; T_3: -13%; Leptin: -45%, Insulin: -44% and Women: IGF-1: -21%; T_3: -10%; Leptin: -43%, Insulin: -38%), with these finding being in line with previous low EA studies in men (Koehler et al., 2016) and women (Ihle & loucks, 2004; Loucks & Thuma., 2003). A limited number of studies have directly assessed sex-related responses to energy deficiency achieved by diet and/or exercise. Some, but not all, previous studies have reported sex differences in regulatory hormones. Short-term (4 days) exercise training in an energy-balanced or energy-deficient state resulted in lower leptin concentrations in women and a trend towards lower insulin concentrations in women compared to men (Hagobian et al., 2009). In the same experiment, there were no sex differences for T_3 concentrations. Leptin concentrations were reduced following 12 weeks of exercise in women, but not in men (Hickey et al., 1997). Similarly, Dubuc et al. (1998) demonstrated marked sex differences in leptin, insulin, glucose and cortisol responses to 7-day energy restriction (Dubuc et al., 1998). In contrast, body weight changes in male and female judoists did not result in a differential leptin response between sexes, with the authors attributing the absence of differences to the high LBM of their female participants, which may have overridden some of the effects of leptin (Prouteau et al., 2006).

When combined the data in women and men, we demonstrated reductions in IGF-1 (-22.4% BASE change), leptin (-44.4% BASE change), T_3 (-11.8% BASE change) and insulin (-22.4% BASE change). These changes are in agreement with previous studies that have shown alterations in regulatory hormones in a direction expected to maintain energy stores

and have correlated regulatory hormones and bone-related changes (Ihle & Loucks, 2004; De Souza et al., 2004; 2010; Zanker & Swaine, 2000). This analysis did not show any changes for sclerostin, GLP-2 or any marker of calcium metabolism in response to low EA; making it unlikely that the alterations in bone turnover shown for data in men and women collectively were mediated by changes in these markers.

Although a comparison of reproductive hormones was not provided in the present study, previous research in physically active women has indicated that low EA alters reproductive hormones (*e.g.*, suppressed LH pulsatility, reduced oestrogen levels) (Loucks & Thuma, 2003) and contributes to the development of reproductive disorders (De Souza, 2003; Williams et al., 2015). These changes may, in turn, influence the bone health of physically active women. Women with menstrual disturbances associated with energy deficiency have altered BTMs (De Souza et al., 2008), lower BMD (Zanker & Swaine, 1998a; De Souza et al., 2008), distorted bone micro-architecture (Ackerman et al., 2011; 2012a) and are at higher risk for stress fracture injuries (Nattiv et al., 2013). Reproductive disturbances including reductions in testosterone (Hackney et al., 1998, Bennell et al., 1996b; Wheeler et al., 1991), elevated SHBG levels, which may reduce the bioavailable testosterone (Dolan et al., 2012), lowered sperm motility and altered sperm quality (De Souza et al., 1994) have been demonstrated in men partaking in weight sensitive sports, however, it remains uncertain, whether reproductive dysfunction in men experiencing energy deficiency is correlated with unfavourable changes in bone health (Tenforde et al., 2016; De Souza et al., 2014b) similar to those seen in physically active women (De Souza et al., 2008). Future studies are needed to investigate the effects of low EA on markers of reproductive function, particularly in men, and their impact on bone metabolism. Studies to compare the contribution of markers of reproductive function to bone metabolism and health in response to low EA in men and women are also required.

To conclude, in this analysis, the data from Study 1 (women-reported in Chapter 4) and Study 2 (men- reported in Chapter 5) were combined to provide comparative insight into the impact of short-term, low EA at 15 $kcal \cdot kgLBM^{-1} \cdot d^{-1}$ on bone metabolism between men and women. Our findings indicated no significant differences between sexes, with the magnitude of BTM responses to short-term low EA (achieved by exercise and dietary energy restriction) being similar in men and women. From a practical perspective, these observed similarities may imply that men and women might benefit equally from preventing low EA.

Future studies are needed to investigate whether the similarities in BTM response to low EA between men and women persist over time.

Chapter 7. Bone metabolic response to short-term low energy availability achieved by diet or exercise individually in physically active eumenorrheic women

7.1. Introduction

Dietary energy restriction and/or exercise are approaches to weight loss utilised by individuals in their attempts to enhance body mass and composition (Loucks et al, 2011; Loucks, 2013). When such strategies are employed, they may affect bone metabolism (Chapter 4; Ihle & Loucks, 2004; Villareal et al., 2016). In our previous studies (Studies 1 and 2, reported in Chapters 4-6), the effects of low EA achieved by combined DEI restriction and EEE on bone metabolism were explored. Low EA can also result from reductions in DEI only or increased EEE independently; however, the effects of reduced EA attained by these modalities on bone metabolism and health have not been previously investigated. We showed that 5 days of reduced EA (through diet and exercise) resulted in a reduction in bone formation synchronously with an increase in bone resorption, in physically active eumeorrheic women (Chapter 4), but not in physically active men (Chapter 5). The importance of EA with direct, but also indirect (through reproductive disturbances) effects on bone health of physically active women has been highlighted by the Female Athlete Triad (Nattiv et al., 2007; De Souza et al., 2014a) and the RED-S models (Mountjoy et al., 2014). Continued research on the effects of low EA on BTM in this population is required to develop effective prevention, early identification and treatment strategies.

Previous studies have compared the effects of energy restriction and weight loss achieved by different methods on bone health; with some of them showing that the bone loss resulting from caloric restriction can be ameliorated with the addition of exercise (Villareal et al., 2006; Ryan et al., 1998; Pritchard et al., 1996), whilst some others demonstrating no benefits from exercise (Svedsen et al., 1993; Nakata et al., 2008). These studies have been conducted in middle-aged (Villareal et al., 2006; Nakata et al., 2008; Pritchard et al., 1996; Rector et al., 2009) or elderly (Armamento-Villareal et al., 2012; Svedsen et al., 1993; Ryan et al., 1998) overweight and obese populations, but no previous study has been performed in normal-weight women during adulthood, which constitutes a critical period for prevention of premature bone loss (Rizzoli et al., 2010). Most trials have compared diet to diet plus exercise, but not diet to exercise alone, and have utilised exercise protocols suitable for overweight rather than normal-weight, young individuals, making it important to explore the effects of energy restriction attained by diet or exercise on bone metabolism in this population.

The modest weight loss rates achieved by exercise compared to diet in long-term studies (>3 months; Soltani et al., 2016) imply poor compliance with exercise regimens, when this is performed out of laboratory conditions, or compensatory eating behaviour, which may lessen the degree of prescribed energy deficit. These methodological limitations underpin the importance of tight experimental control when comparing the effects of energy restriction of different origin (diet- or exercise- induced) on bone metabolism and health. The design and implementation of an energy deficit based on EA over the traditionally used energy balance is advantageous in prescribing dietary and exercise regimens (Loucks, 2013). Exploring the acute effects of energy restriction (rather than chronic) also allows stringent control of exercise training and DEI prescription.

Alterations in regulatory and reproductive hormones due to energy deprivation may contribute to changes in bone metabolism (De Souza et al., 2008; Ihle & Loucks, 2004). Responses in some regulatory hormones have been reported to differ during exercise and dietary restriction. Decreases in PYY and increases in ghrelin levels have been previously reported following severe dietary energy deprivation, but no compensatory alterations occur after exercise-induced energy deficit (King et al., 2011; Alajmi et al., 2016). In contrast, some other regulatory hormones including leptin and insulin, appear to be similarly altered in energy deprivation independent of modality of implementation (Koelher et al., 2016). If these changes following diet- or exercise-induced energy deficit are related to changes in bone metabolism remains unexplored. Anorexia nervosa and exercise-induced amenorrhoea suggest that low EA, regardless of origin, may similarly result in severe oestrogen deficiency with negative consequences in bone health (Misra, 2012). Observational studies suggest that the degree of compromised bone health may be more severe in women with amenorrhea resulting from endocrine disturbances or prolonged undernutrition (e.g., anorexia nervosa) compared to exercise–induced amenorrhea (Cann et al., 1984). However, a systematic approach to simultaneously determine changes in BTM and reproductive hormones in response 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 determine and compare the effects of reduced EA at 15 kcal·kg $LBM^{-1}\cdot d^{-1}$ induced by diet alone or exercise alone on BTM in physically active, eumenorrheic women. This study explored responses of regulatory and reproductive hormones and markers of calcium metabolism to diet- and exercise-induced low EA that

may be related to BTM responses. It was hypothesised that diet-and exercise-induced low EA would decrease bone formation and increase bone resorption.

7.2. Methods

7.2.1. Participants

Ten eumenorrheic women (Table 7.1) volunteered to participate in this experiment after providing informed written consent (Appendix 12-Paticipant's Information Sheet, Appendix 13-Informed Consent Form). Participants were required to be aged 18-40 years, non-smokers, currently injury free and have a BMI between 18.5 and 30 $kg \cdot m^{-2}$. Participants were excluded from partaking in the study if they were using any medication or suffering from any condition known to interfere with bone metabolism or if they had sustained a bone fracture within the previous year. They were also excluded if they were breastfeeding, pregnant, using any type of hormonal contraception or self-reported short (<24 days), long (>35 days) or irregular menstrual cycles (please see section 3.3.2.). These criteria were confirmed verbally and in writing via a health screen (please see section 3.3.1.). Participants regularly performed ≥3 hours of moderate to vigorous physical activity per week and had moderate and high physical activity levels as determined by the IPAQ (please see section 3.3.4.).

7.2.2. Experimental design

The study utilised a randomised, crossover, design, in which participants completed 3 experimental conditions, an energy-balanced, controlled EA (CON), a diet-induced restricted EA (D-RES) and an exercise-induced restricted EA (E-RES) in a counterbalanced fashion. Participants attended an initial preliminary assessment (P) and a 3-day habitual assessment (H1-H3) prior to completing the 3 experimental conditions. Each condition began with the identification of the beginning of their menstrual cycle (D1) followed by a 5-day experimental period (D2-D6). The controlled EA was set at 45 $kcal \cdot kg\ LBM^{-1} \cdot d^{-1}$ and achieved without exercise. Both restricted EA (E-RES and D-RES) were administered as 15 $kcal \cdot kg\ LBM^{-1} \cdot d^{-1}$, with this being achieved by diet only in D-RES and by exercise only in E-RES. In D-RES, participants refrained from any exercise and consumed a diet providing them with 15 $kcal \cdot kg\ LBM^{-1} \cdot d^{-1}$. In E-RES, participants completed exercise sessions at an exercise intensity of 70% of their VO_2 peak that resulted in an EEE of 30 $kcal \cdot kg\ LBM^{-1} \cdot d^{-1}$. Their DEI was controlled at 45 $kcal \cdot kg\ LBM^{-1} \cdot d^{-1}$ in order to achieve the same EA of 15 $kcal \cdot kg\ LBM^{-1} \cdot d^{-1}$ as in D-RES (please see Figure 7.1.). There was an approximately 28-day gap between the completion of one experimental period and the beginning of the next

experimental period, meaning that all testing was completed during the same phase of the menstrual cycle (early follicular).

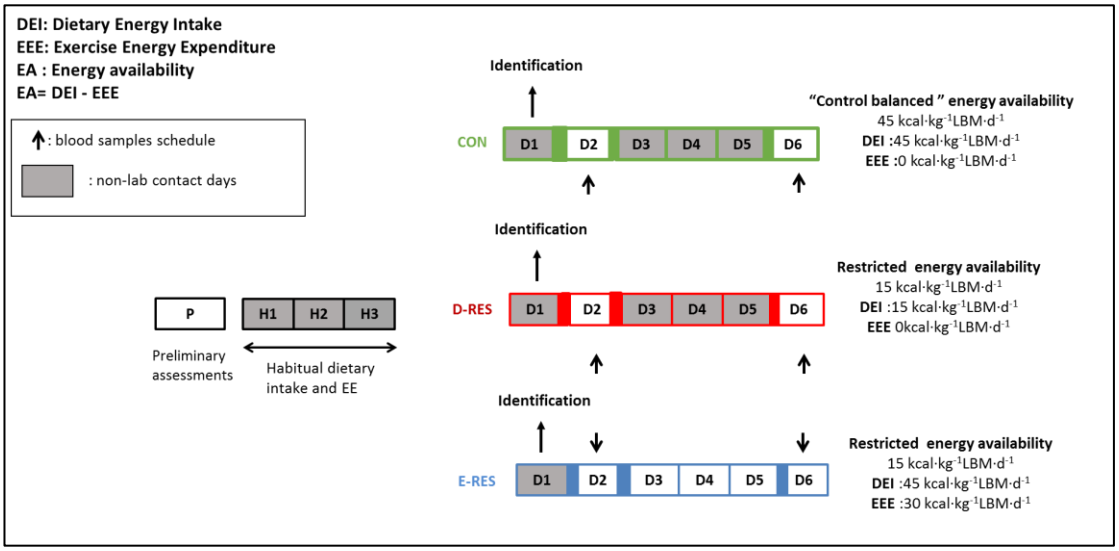


Figure 7.1. Overview of the study design. Preliminary day (P) and habitual dietary and exercise assessment (H1-H3) were performed only once before the controlled (CON), diet-induced restricted EA (D-RES) or exercise-induced restricted EA (E-RES). D1: Day 1 of bleeding and identification for experimental protocol initiation, D2-D6: Experimental Days, the thick green/red/blue lines denote change of condition; 1st thick line-transition from identification to the experimental protocol; 2nd thick line-initiation of CON/D-RES/E-RES; 3rd thick line- end of experimental condition.

7.2.3. Experimental procedures

7.2.3.1. Preliminary assessments and 3-day habitual assessment

A preliminary visit was performed to establish inclusion criteria, take baseline measurements and determine the fitness level of the volunteers. These procedures have been previously described in section 4.2.3.1. In this study, whole body DXA scans were performed and analysed by the same operator at Nottingham Trent University. Positioning of the participants during the DXA scan was performed according to manufacturer's guidelines, as detailed in section 3.5. Similar to previous studies, participants performed an incremental exercise test on a treadmill (HP Cosmos, Germany) to determine their VO_2 peak (please see section 3.3.6). for a full description. All participants were provided with

accelerometers and food weighing scales (please see 3.7.1. and 3.8.1.) in order to record lifestyle EE and habitual DEI for 3 days (H1-H3; Figure 7.1.).

7.2.3.2. Experimental period

Participants notified the researcher at the onset of their menstrual cycle (D1), which indicated the first day of the experimental period. On the next morning (D2), a blood sample was collected and used as the baseline (BASE) sample prior to each experimental condition. The following three days of the protocol (D3-D5) were the experimental condition days. Over D3-D5, participants undertook CON, E-RES and D-RES in a counterbalanced order. On D6, participants had a follow-up blood sample (Figure 7.1.).

Due to scheduling constraints, such as availability of participants or laboratories, 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, please consider that we will refer to D2 as BASE, D3-D5 as the main experimental period and D6 as the follow-up (Figure 7.1.).

7.2.3.2.1. Lifestyle EE

Participants wore an accelerometer (GT3X/GT3XE, Actigraph) during all waking hours, except while bathing, to estimate lifestyle EE. Please also see section 4.2.3.2.1.

7.2.3.2.2. Habitual DEI

Participants weighed and recorded food intake during H1-H3 to provide information about their habitual DEI. Please also see section 4.2.3.2.2.

7.2.3.2.3. Experimental diets

In CON, D-RES and E-RES participants consumed diets providing 45, 15 and 45 $kcal \cdot kgLBM^{-1} \cdot d^{-1}$. 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. Acceptability of food items to be provided throughout the experimental period was tested verbally and participants had to choose up to 3 out of 4 available menus (Appendix 14). Each menu included 5 meals in all experimental conditions to limit the effects of food partition on bone turnover (Li & Muhlbauer, 1999). A registered

dietitian designed menus 1-4 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 3 in D-RES (15 kcal·kgLBM^{-1}·d^{-1}), but were unchanged in E-RES condition (45 kcal·kgLBM^{-1}·d^{-1}). For participants with a different LBM than the reference individual, food quantities in all menus and conditions were multiplied by a scaling factor to account for the differences in LBM compared to the reference individual. All meals and snacks were weighed to the nearest 1g (Electronic Kitchen Scale, Argos, UK) and packaged by the study investigators (Figure 7.2). Participants were encouraged to consume only the pre-packaged meals and snacks for the 3 days of main experimental protocol in an effort to increase compliance to the experimental diets. Participants were allowed to follow their habitual tea and coffee drinking without any sugar or milk. Adherence to these experimental conditions was verbally confirmed with the participants at various points throughout the protocol. A multivitamin, multi-mineral supplement (A - Z Tablets, Boots, Nottingham, UK) was provided during D-RES only in order to provide adequate micronutrient intake and isolate the effects of energy/macronutrient restriction.

7.2.3.2.4. EEE

In E-RES only, participants completed exercise sessions that resulted in EEE of 30 kcal·kgLBM^{-1}·d^{-1}. Participants ran on a flat treadmill while being continuously supervised. Exercise intensity was controlled by setting treadmill speed to achieve 70% of VO_2 peak for each participant. On the first day of the exercise protocol (D3, E-RES) participants wore a facemask throughout the exercise in order to measure EEE. The procedures of testing and the calculation of EEE have been described in detail (please see section 3.8.2.). In order to increase compliance, the total duration of the exercise per day was split up in 2 sessions of equal duration. EEE resulting of exercise of the same duration and intensity is consistently similar when repeated under the same conditions for the same individual, as shown in section 3.8.3. As such, gas analysis was not performed during the remainder of the exercise sessions (D3 afternoon-D5).

7.2.4. Storage and analyses of blood samples

Blood samples were obtained at the same time of day for each participant between 07:30-08:15 h (± 10 min for the same participant) after an overnight fast (from 20:00 h the previous evening) on D2 (BASE) and D6. Blood sample collection, processing and analysis have been

previously described in section 3.1.3. β-CTX, PINP, PTH and IGF-1 were analysed in plasma. Serum was analysed for leptin, insulin, T_3, 17β-oestradiol, ACa, Mg and PO_4.

7.2.5. Biochemical analysis

Detailed description on biochemical analysis has already been provided in section 3.13.

7.2.6. Measures of cognitive function and muscle function

Participants also undertook measures of cognitive function and muscle function. The cognitive function test battery was performed during the preliminary visit and on D2 and D6 of the experimental protocol for the three experimental conditions. The cognitive test battery was conducted in the same order each session and included the Rey Auditory Verbal Learning test, Mental Rotation Test, Visual Search, Stroop Test and Rapid Visual Information Processing, followed by a delayed recall secondary component of the Rey Auditory Verbal Learning test. Muscle function tests were performed during the preliminary visit and on D2 and D6 of the experimental protocol for the three experimental conditions. Maximal voluntary isometric force was assessed for quadriceps and first dorsal interosseus muscle using custom built dynamometers immediately after the cognitive function tests. The results of these measurements are not presented here and were for another PhD thesis.

7.2.7. Statistical analysis

Sample size calculations indicated that testing 7 participants would allow detection of a significant change in P1NP (pre: 76.1 ± 5.8; post: 64.7± 6.0 $mg \cdot L^{-1}$, $P<0.05$) following low EA (Zanker and Swaine, 2000) at P=0.05 and power = 0.80. All data were checked for normality according to the Shapiro-Wilk test and non-parametric tests or logarithmic transformations were employed as necessary to ensure the validity of statistical analysis. Baseline biochemistry and markers of energy status prior to each experimental condition were compared with one-way repeated measures ANOVA. 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 BTMs, regulatory hormones, markers of calcium metabolism and reproductive hormones. Area under the curve (AUC) with respect to baseline (BASE) was calculated for all biochemical markers from the data expressed as %BASE (Zweig and Campbell, 1993). One-way repeated measures ANOVA was used to assess differences in all outcomes expressed as AUC between the 3 experimental conditions. Significant main or interaction effects were followed by Tukey's *post-hoc* analysis. Data are

presented as mean (1SD) and statistical significance was set at $P \leq 0.05$. Data were analysed using Statistica 13.0 (Statsoft, USA). In addition to summary statistics, the individual responses of the BTM to D-RES and E-RES were also examined. In order to be considered a responder, β-CTX concentrations at D6 in D-RES and 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 RES were <BASE (100%), <P1NP levels at D6 in CON together with a difference >3% to account for CV for P1NP assay.

7.3. Results

7.3.1. Participant characteristics

Participants were young, healthy, normal-weight and physically active (Table 7.1.).

Table 7.1. Participant characteristics (n=10).

Demographics	
Age (y)	24 (3)
Height (m)	1.66 (0.05)
Body mass (kg)	61.06 (6.97)
BMI (kg ·m^{-2})	22.3 (2.4)
Body composition	
Body fat (%)	29.3 (5.1)
LBM (kg)	41.3 (4.1)
FFM (kg)	44.3 (4.3)
BMD (g·cm^{-2})	1.19 (0.09)
Training characteristics	
VO_2 peak (ml·kg^{-1}·min^{-1})	48.1 (3.3)
VO_2 peak (ml·kg LBM^{-1}·min^{-1})	70.9 (2.8)
Physical activity (MET-min·week^{-1})	4634 (2382)
Dietary and EE characteristics	
Habitual DEI (kcal·d^{-1})[1]	1999 (371)
Lifestyle EE (kcal·d^{-1})[1]	422 (123)
24-hour EE(kcal·d^{-1})[1]	2052 (197)

Values are expressed as means (1SD).
[1]Analysis performed in 9 partictipants with complete data.
BMI: Body mass index; LBM: Lean body mass; FFM: Fat free mass; BMD: Bone mineral density: VO_{2max}: Maximum oxygen uptake; DEI: dietary Energy Intake; EE: Energy expenditure; RMR: Resting metabolic rate; LBM: Lean body mass; MET: Metabolic equivalents.

7.3.2. Baseline Biochemistry

There were no significant differences in any biochemical marker prior to CON, D-RES and E-RES (Table 7.2.).

Table 7.2. Markers of energy status and BASE concentrations of BTMs, markers of calcium metabolism, regulatory and reproductive hormones prior to CON, D-RES and E-RES (n=10)

	CON	D-RES	E-RES
Markers of energy status			
Body mass (kg)	60.9 (7.0)	61.5 (7.0)	61.1 (6.3)
BTMs			
***β*-CTX (μg·L^{-1})**	0.50 (0.19)	0.48 (0.18)	0.47 (0.24)
P1NP (μg·L^{-1})	56.7 (16.9)	54.8 (12.7)	55.3 (14.4)
BT ratio	1.19 (0.24)	1.21 (0.26)	1.30 (0.39)
Markers of calcium metabolism			
PTH (pg·mL^{-1})	4.4 (1.1)	4.0 (0.9)	4.6 (1.4)
ACa (mmol·L^{-1})	2.30 (0.05)	2.27 (0.03)	2.29 (0.04)
Mg (mmol·L^{-1})	0.83 (0.02)	0.81 (0.03)	0.81 (0.03)
PO_4 (mmol·L^{-1})	1.29 (0.12)	1.26 (0.14)	1.33 (0.15)
Regulatory hormones			
IGF-1 (mmol·L^{-1})	205.0 (39.4)	202.5 (46.8)	220.6 (56.7)
T_3 (mmol·L^{-1})	1.49 (0.34)	1.53 (0.28)	1.53 (0.31)
Leptin (ng·mL^{-1})	6.7 (4.1)	7.1 (4.1)	6.0 (3.3)
Insulin (pmol·L^{-1})	31.6 (7.7)	33.8 (9.0)	45.6 (34.8)
Reproductive hormones			
17-*β* oestradiol (pmol·L^{-1})	108.9 (33.6)	118.9 (29.7)	148.3 (92.9)

Values are expressed as means (1SD).
β-CTX: C-terminal cross-linked telopeptides of type I collagen; P1NP: Amino-terminal pro-peptides of type 1 procollagen; BT ratio: Bone turnover ratio; 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; BTM: Bone turnover marker; CON: Controlled; D-RES: Diet-induced restricted trial; E-RES: Exercise-induced restricted trial.

7.3.3. Baseline energy status and reproductive function

There were no differences in body mass prior to CON, D-RES and E-RES (Table 7.3.). Women began each condition in the early follicular phase, as confirmed by *17β*-oestradiol at BASE in all experimental conditions (Stricker et al., 2006) (Table 7.2). *17β*-oestradiol concentrations for one of the participants in the E-RES trial indicated that she was approaching or experiencing ovulation (please also see section 7.2.2); therefore, this participant was removed prior to final statistical analysis (n=10).

7.3.4. Body mass

There was no significant reduction in body mass in CON (BASE: 60.9±7.0kg, D6:60.3± 6.7 kg, P=0.053). Body mass significantly decreased from BASE in D-RES (BASE: 61.4±6.8, D6: 59.6±6.5, P<0.001) and E-RES (D6: 61.1±6.3- 60.1±6.0, 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.

7.3.5. BTMs

7.3.5.1. *β*-CTX

There was a significant main effect of time for *β*-CTX (P=0.044), with higher mean concentrations at D6 compared to BASE. No significant main effect of condition (P=0.13) or any condition x time interaction effect (P=0.17) was shown for *β*-CTX concetrations (Figure 7.2.; Table 7.3.). *β*-CTX AUC was not significantly different between CON, D-RES and E-RES (P=0.16) (Figure 7.3.; Table 7.3.).

7.3.5.2. P1NP

There was a significant main effect of time for P1NP concentrations (P<0.001), with reduced mean concentrations at D6 compared to BASE. There was no significant condition effect (P=0.25) or any condition x time interaction effect (P=0.052) (Table 7.5.; Figure 7.2.). P1NP AUC was not significantly different between CON, D-RES and E-RES (condition effect, P=0.054) (Figure 7.3; Table 7.3.).

7.3.5.3. BT ratio

A significant effect of time (BASE< D6) was shown for BT ratio (P=0.003), but no main effect of condition (P=0.21) or any condition x time interaction effect (P=0.10). BT Ratio AUC was significantly lower after D-RES compared to CON only (condition effect; P=0.041) (Figure 7.4; Table 7.3.).

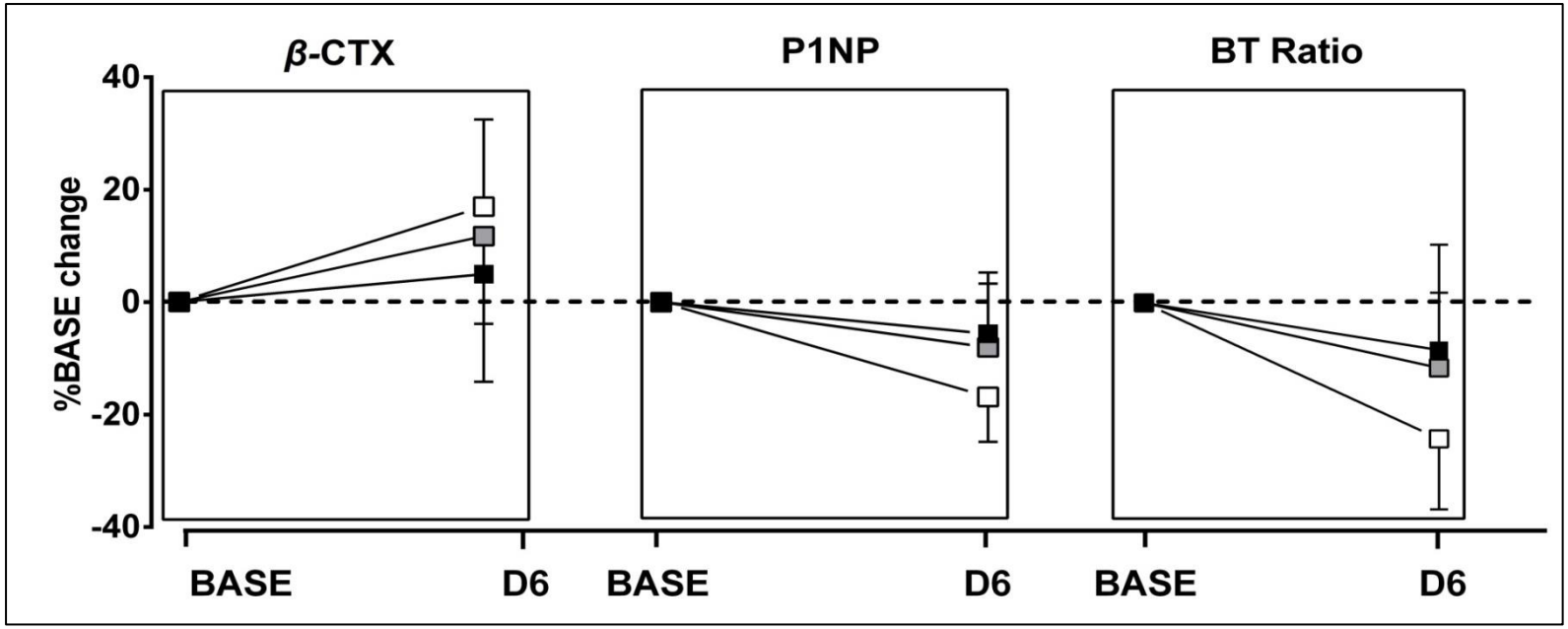


Figure 7.3. Percentage change from BASE concentrations for *β*-CTX, P1NP and BT ratio on D6 in CON (black squares) and D-RES (white squares) and E-RES (grey squares) (n=10). Values are presented as means (1SD). *β*-CTX: C-terminal cross-linked telopeptide of type I collagen; P1NP: Amino-terminal pro-peptides of type 1 procollagen; BT ratio: Bone turnover ratio; CON: Controlled trial; D-RES: Diet-induced E-Exercise-Induced Restricted trial; D: Day BASE: Baseline; CON: Controlled; D-RES: Diet-Induced Restricted Trial; E-RES: Exercise-Induced Restricted Trial.

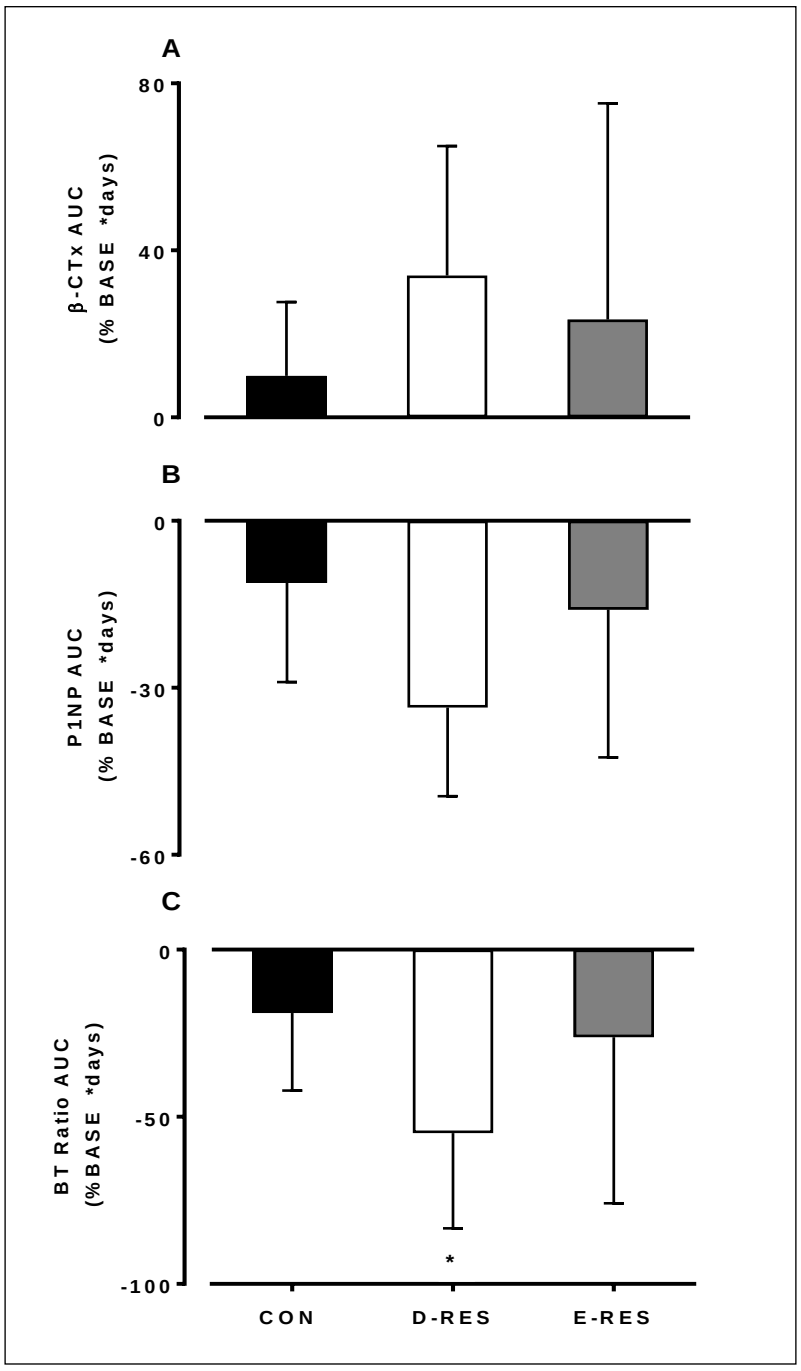


Figure 7.4. AUC analysis of *β*-CTX (A), P1NP (B) and BT ratio (C) after CON (black bars) and D-RES (white bars) and E-RES (grey bars) (n=10). Values are presented as means (1SD). $^{*}P<0.05$, denotes a significant difference from CON. *β*-CTX: C-terminal cross-linked telopeptide of type I collagen; P1N: Amino-terminal pro-peptide of type 1 procollagen; BT ratio: Bone turnover ratio; BASE: Baseline; AUC: Area under the curve; CON: Controlled trial; D-RES: Diet-induced restricted trial; E-RES: Exercise-induced trial.

Table 7.3. BTMs expressed as concentrations, percentage change from BASE and AUC in CON, D-RES and E-RES trials (n=10). Values of D2 were used as BASE prior to each experimental condition.

	CON		D-RES		E-RES	
	BASE	**D6**	**BASE**	**D6**	**BASE**	**D6**
***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)
%BASE change		5.0 (8.8)		17.0 (15.5)		11.7 (25.8)
AUC (%BASE x d)[1]		10.0 (17.7)		34.0 (31.0)		23.5 (51.8)
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)
%BASE change		-5.6 (8.9)		-16.8 (8.0)		-8.0 (13.0)
AUC (%BASE x d)[1]		-11.1 (17.8)		-33.5 (16.0)		-16.0 (26.5)
BT ratio						
-	1.19 (0.24)	1.06 (0.20)	1.21 (0.26)	0.85 (0.14)	1.30 (0.39)	1.06 (0.23)
%BASE change		-9.5 (12.0)		-27.4 (11.3)		-13.1 (24.9)
AUC (%BASE x d)[1]		-19.0 (23.2)		-54.9 (28.5)*		-26.2 (49.7)

Values are expressed as means (1SD).
[1]AUC calculated for each experimental condition from BASE to Day 6.
*denotes a significant difference from CON ($P<0.05$).
β-CTX: C-terminal cross-linked telopeptide of type I collagen; P1NP: Amino-terminal pro-peptide of type 1 procollagen; BT ratio: Bone turnover ratio; BTM: Bone turnover marker; D: Day; BASE: Baseline; AUC: Area under the curve; CON, Controlled trial; D-RES, Diet-Induced Restricted trial; E-RES: Exercise-Induced Restricted trial.

7.3.6. Markers of calcium metabolism

7.3.6.1. PTH

There was a significant main effect of time for PTH (P=0.002), with lower levels at D6 compared to BASE. There was no significant main effect of condition (P=0.21) or any condition x time interaction effect (P=0.90). PTH AUC in CON was not significantly different from AUC in either D-RES or E-RES (condition effect, P=0.95) (Table 7.4.).

7.3.6.2. ACa

There was no significant main effect of time (P=0.41), condition (P=0.07) or any condition x time interaction effect (P=0.10) for ACa levels. ACa AUC was not significantly different between experimental conditions (P=0.10) (Table 7.4.).

7.3.6.3. Mg

There was no significant main effect of time (P=0.51), condition (P=0.54) or any condition x time interaction effect (P=0.89) for Mg concentrations. Mg AUC was not significantly different between CON, D-RES and E-RES (P=0.89) (Table 7.4.).

7.3.6.4. PO_4

There was no significant main effect of time (P=0.22) or condition (P=0.76). A condition x time interaction effect (P=0.01) was shown for PO_4 concentrations, with PO_4 concentrations decreasing by -9.4% from BASE in E-RES trial only. PO_4 AUC was significantly lower in E-RES compared to D-RES only (P=0.01) (Table 7.4.).

Table 7.4. Markers of calcium metabolism expressed as concentrations, percentage change from BASE and AUC in CON, D-RES and E-RES trials (n=10). Values of D2 were used as BASE prior to each experimental condition.

	CON		D-RES		E-RES	
	BASE	**D6**	**BASE**	**D6**	**BASE**	**D6**
PTH						
pg·mL^{-1}	4.37 (1.13)	3.78 (0.49)	4.03 (0.92)	3.69 (0.69)	4.60 (1.44)	4.09 (0.78)
%BASE change		-8.0 (26.4)		-4.3 (26.5)		-4.3 (31.4)
AUC (%BASE x d)[1]		-15.6 (52.8)		-8.5 (52.9)		-8.6 (62.8)
ACa						
mmol·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)
%BASE change		0.8 (2.1)		0.0 (2.1)		-1.4 (1.4)
AUC (%BASE x d)[1]		1.5 (4.2)		-0.7 (4.2)		-2.7 (2.8)
Mg						
mmol·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)
%BASE change		-0.7 (2.3)		-0.7 (7.0)		0.4 (5.4)
AUC (%BASE x d)[1]		-1.5 (4.6)		-1.4 (14.0)		0.9 (10.7)
PO_4						
mmol·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)[*]
%BASE change		-0.5 (8.6)		4.6 (11.0)		-9.4 (10.3)
AUC (%BASE x d)[1]		-0.9 (17.2)		9.3 (21.9)		-18.8 (20.5)[**]

Values are expressed as means (1SD).

[1]AUC calculated for each experimental condition from BASE to Day 6.

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

[**]denotes a significant difference from D-RES (P<0.05).

PTH: Parathyroid hormone; Mg: Magnesium; ACa: Albumin adjusted Calcium; PO_4: Phosphate; D: Day; BASE: Baseline; AUC: Area under the curve; CON, Controlled trial; D-RES, Diet-Induced Restricted trial; E-RES: Exercise-Induced Restricted trial.

7.3.7. Regulatory hormones

7.3.7.1. IGF-1

A significant main effect of time was shown for IGF-1 (P=0.01); concentrations at D6 decreased from BASE. There was a significant main effect of condition (E-RES<CON; P=0.03) and a significant condition x time interaction effect (P<0.001). IGF-1 concentrations at D6 in D-RES and E-RES decreased by 13.2% (P=0.01) and 23.4% (P<0.001) from BASE and were both significantly lower than IGF-1 concentrations at D6 in CON (P<0.001). IGF-1 AUC was lower in D-RES (P<0.001) and E-RES (P<0.001) compared to CON (Table 7.8.), but there were no significant differences between D-RES and E-RES (P=0.12) (Table 7.5.).

7.3.7.2. T_3

There was no significant main effect of condition (P=0.11), but a significant main effect of time (BASE<D6; P=0.01) and a condition x time interaction (P=0.03) were shown for T_3 concentrations. T_3 concentrations at D6 in D-RES were significantly decreased by 14.8% from BASE (P=0.002) and were lower than the concentrations in CON at the same time-point (P=0.02). T_3 AUC in D-RES was lower compared to CON (P=0.02), but not significantly different compared to E-RES (P=0.25) (Table 7.5.).

7.3.7.3. Insulin

There was a significant main effect of time (BASE<D6; P=0.03) and a significant time x condition interaction effect (P=0.04), with insulin concentrations at D6 in E-RES decreasing by 36.5% from BASE. No significant main effect of condition (P=0.31) was shown for insulin concentrations. Insulin AUC were not significantly different between CON, D-RES and E-RES (P=0.13) (Table 7.5.).

7.3.7.4. Leptin

There was a significant main effect of time (D6<BASE; P<0.001) and condition (P=0.004), with leptin concentrations in D-RES (P=0.006) and E-RES (P=0.02) being lower than CON. A time x condition interaction effect was also shown; leptin concentration at D6 in CON, D-RES and E-RES were significantly decreased by 30.0% (P=0.04), 59.0% (P<0.001) and 60.5% (P<0.001) from BASE prior to each experimental condition. Concentrations at D6 in D-RES (P<0.001) and E-RES (P<0.001) were also different from concentrations at D6 in CON. Leptin AUCs in D-RES (P=0.002) and E-RES (P=0.001) were significantly lower

than CON, but there were no significant differences between D-RES and E-RES (P=0.97) (Table 7.5.).

7.3.8. Reproductive hormones

There was a significant main effect of time for 17*β*-oestradiol levels (P=0.002) with higher levels at D6 compared to BASE, which is in line with the progression of the menstrual cycle. No main effect of condition (P=0.47) or any condition x time interaction (P=0.30) were shown for 17*β*-oestradiol levels. AUC for 17*β*-oestradiol was not significantly different between conditions (P=0.24) (Table 7.5.).

7.3.9. Individual analysis

Individual responses for *β*-CTX and P1NP, as well as altered bone turnover due to increased *β*-CTX, decreased P1NP or both, in D-RES and E-RES trials are presented in Table 7.6.

Table 7.6. Number of responders (out of total number of participants) for *β*-CTX, P1NP and bone turnover in D-RES and E-RES. This analysis was based on data expressed as % BASE for each participant.

***β*-CTX**		**P1NP**		**Bone turnover[1]**		**Bone turnover[2]**	
D-RES	**E-RES**	**D-RES**	**E-RES**	**D-RES**	**E-RES**	**D-RES**	**E-RES**
5/10	4/10	5/10	3/10	8/10	5/10	2/10	2/10

[1]altered bone turnover due to increased *β*-CTX, decreased P1NP or both procedures.
[2]altered bone turnover due to a simultaneous increase in *β*-CTX and decrease in P1NP.
β-CTX: C-terminal cross-linked telopeptide of type I collagen; P1NP: Amino-terminal pro-peptides of type 1 procollagen; BT ratio; BASE: Baseline; AUC: Area under the curve; CON, Controlled trial; D-RES, Diet-induced restricted trial; E-RES: Exercise-induced restricted trial.

Table 7.5. Regulatory and reproductive hormones expressed as concentrations, percentage change from BASE and AUC in CON, D-RES and E-RES trials (n=10). Values of D2 were used as BASE prior to each experimental condition.

	CON		D-RES		E-RES	
	BASE	**D6**	**BASE**	**D6**	**BASE**	**D6**
IGF-1						
mmol·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)$^{*, **}$
%BASE change		10.6 (18.9)		-13.2 (9.1)		-23.4 (9.5)
AUC (%BASE x d)[1]		21.3 (37.9)		-26.4 (18.2)**		-46.7 (19.1)**
T_3						
mmol·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)
%BASE change		0.3 (11.0)		-14.8 (11.2)		-6.9 (11.0)
AUC (%BASE x d)[1]		0.5 (22.6)		-29.5 (22.4)**		-13.7 (22.1)
Leptin						
ng·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)$^{*, **}$
%BASE change		-30.0 (22.7)		-59.0 (19.6)		-60.6 (16.7)
AUC (%BASE x d)[1]		-60.0 (45.4)		-118.0 (39.1)**		-121.3 (33.4)**
Insulin						
pmol·L^{-1}	31.6 (7.7)	36.7 (20.9)	33.8 (9.0)	28.6 (15.0)	45.6 (34.8)	20.4 (11.3)$^{*, **}$
%BASE change		13.0 (44.0)		-6.4 (62.5)		-36.5 (49.3)
AUC (%BASE x d)[1]		26.0 (87.9)		-12.8 (125.0)		-73.0 (98.5)**
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)
%BASE change		48.3 (35.2)		32.2 (39.2)		24.0 (39.9)
AUC (%BASE x d)[1]		96.6 (70.4)		64.5 (78.4)		48.1 (79.8)

Values are expressed as means (1SD).
[1]AUC calculated for each experimental condition from BASE to Day 6.
*denotes a significant difference between BASE and D6 ($P<0.05$).
**denotes a significant difference from CON at the same time point ($P<0.05$).
T_3: Triiodothyronine; IGF-1: Insulin-like growth factor 1; D: Day; BASE: Baseline; AUC: Area under the curve; CON: Controlled trial; D-RES, Diet-induced restricted trial; E-RES: Exercise-induced restricted trial.

7.4. Discussion

This study yielded 3 main findings 1) neither diet-induced nor exercise-induced low EAs resulted in changes in markers of bone formation or bone resorption, but a significantly reduced BT ratio was shown in the diet-induced low EA only, 2) there were no differences in BTM responses between diet- and exercise-induced low EAs and 3) both reduced EAs were accompanied by reductions in IGF-1 and leptin; T_3 was reduced following diet-induced low EA only and insulin decreased in exercise-induced low EA only. These findings suggest that low EA attained by diet or exercise does not affect bone formation or resorption within the 3-day timeframe of the present study, despite changes in regulatory hormones.

Bone formation (-17% from BASE in P1NP) and resorption (+17% from BASE in β-CTX) responses to a 3-day diet-induced low EA did not reach statistical significance. BT ratio was significantly lower (-27% from BASE) compared to the controlled condition. In a previous experiment, bone formation was significantly reduced after 4 days of fasting, as indicated by decreases in P1CP and OC by 45% and 58% (Grinspoon et al., 1995). In the same experiment, bone resorption assessed by urinary PYD and DPD also decreased to a similar extent. The authors suggested simultaneous alterations in bone formation and resorption (Grinspoon et al., 1995). Direct comparisons of the magnitude of the effects between the two studies cannot be performed due to the differences in BTMs used to measure bone metabolic activity. P1NP was measured, which is the reference standard for bone formation (Vasikaran et al., 2011) and may better reflect bone formation compared to P1CP (possibly due to differences in the regulation of their catabolism) and OC (reflective of overall alterations in bone turnover rather than bone formation alone) (Lombardi et al., 2012a). Similarly, β-CTX has been proposed as the reference standard for bone resorption (Vasikaran et al., 2011) and is superior to urine PYD and DPD measurements, which are limited by sample collection (*i.e.*, second void or 24h sampling) and creatinine corrections (Vasikaran et al., 2011). In addition to these analytical differences, our dietary prescription represented a 67% energy deficit imposed over a 3-day period, which is less severe than complete food deprivation over 4 days.

In Study 1 (reported in Chapter 4), low EA at 15 kcal·kgLBM^{-1}·d^{-1}, achieved by a combination of dietary energy restriction and exercise, significantly increased bone resorption (β-CTX AUC) and reduced bone formation (P1NP AUC) and BT ratio (AUC) following a 5-day experimental protocol in physically active eumenorrheic women. In the follow-up blood sample after 3 days of low EA, in Study 1, there was a 19% increase in β-

CTX and a 14% and 26% reduction in P1NP and BT ratio from BASE. The direction and magnitude of these changes were similar with the results shown for the diet-induced reduced EA of the present study (*β*-CTX: +17%, P1NP: -17%, BT ratio: -27%), suggesting that we cannot exclude significant changes in BTM response to diet-induced low EA, if a longer protocol was used.

Exercise-induced low EA at 15 kcal·kg $LBM^{-1}\cdot d^{-1}$ did not alter bone formation (-8% from BASE in P1NP), bone resorption (+12% from BASE in *β*-CTX) or BT ratio (-13% from BASE). From a practical point of view, our exercise intervention was representative of 2-2.5 h of running at moderate intensity (70% VO_2 peak), which is a common training programme followed by some athletic and military populations (Tharion et al., 2005; Pontzer, 2015). Our study goes beyond the sedentary population used in previous short-term experimental studies on EA and BTM (Ihle & Loucks, 2004) by including physically active women, able to perform strenuous exercise. However, our participants were not accustomed to such a prolonged duration of daily exercise over consecutive days. The responses shown might reflect some osteogenic effects due to unusual, high impact mechanical loading (Robling et al., 2006; Bonnet & Ferrari, 2010), which may have masked some of the effects of low EA, especially at weight bearing sites. Notably, BTMs are systematic in the circulation and provide information about overall bone turnover. As such, we were unable to detect potential changes in specific skeletal sites (*i.e.*, non-weight bearing sites).

When we compared BTMs in response to diet-induced and exercise-induced low EAs, there were no significant differences between conditions within the 3-day experimental period. Individual analysis of BTM responses to diet-induced and exercise-induced low EAs showed a more consistent BTM response to diet-induced low EA compared to exercise-induced low EA; with 8 out of 10 in the diet-induced low EA trial and 5 out of 10 participants in the exercise-induced low EA trial having altered bone turnover due to increase *β*-CTX, decreased P1NP or both. It is uncertain if the similarities in BTM responses shown for diet-induced and exercise-induced low EAs persist over time or whether we were unable to capture any differences due to the short duration of our experimental protocol. Taken together, these results suggest that a subset of women adversely responded to low EA irrespective of origin (diet or exercise), suggesting that individual factors might influence the susceptibility to low EA from a bone health point of view in women (Female Athlete Triad, Nattiv et al., 2007; De Souza et al., 2014a and RED-S models, Mountjoy et al., 2014; 2015).

Evidence on the effects of exercise and energy restriction on bone metabolism and health are unclear. Some studies in overweight and obese individuals have reported bone loss

regardless of the weight loss method used (diet compared to either exercise or diet and exercise) (Svedsen et al., 1993; Nakata et al., 2008). In contrast, some others have shown a protective effect of exercise on BMD compared to diet-induced weight loss (Villareal et al., 2006; Ryan et al., 1998; Pritchard et al., 1996). A recent meta-analysis concluded that caloric restriction results in bone loss in weight-bearing skeletal sites (hip, lumbar spine), whereas exercise-induced weight loss does not (Soltani et al., 2016). There are a number of challenges in studies of overweight and obese individuals, including difficulty in performing exercise of sufficient intensity to impact bone metabolism, poor compliance with exercise protocols, initial increased body mass and different weight loss rates in diet- and exercise-induced energy restriction. These factors may not apply in lean and more physically active populations, limiting the usefulness and transferability of existing findings in overweight and obese populations to physically active populations.

In non-overweight individuals, the osteoprotective effects of exercise in energy-restricted conditions may be more pronounced. Observational studies have shown that bone loss associated with exercise–induced amenorrhea is less than that experienced by women with amenorrhea due to endocrine disturbances or anorexia nervosa (Cann et al., 1984). These findings support the notion that mechanical loading has some beneficial effects on the skeleton, which counteract some of the unfavourable effects of amenorrhea (Borer, 2005). Further evidence suggests that dancers with amenorrheea have greater BMD at weight bearing sites (*e.g.*, proximal femur, lumbar spine) compared to girls with anorexia nervosa with similarly low body mass (Young et al., 1994). Conversely, similar bone loss occurs in amenorrheic athletes and individuals with anorexia nervosa at non-weight bearing skeletal sites (Young et al., 1994). Athletes participating in weight sensitive, non-weight bearing sports such cycling are at a greater risk for developing osteopenia or osteoporosis than those partaking in weight bearing activities (Rector et al., 2009; Dolan et al., 2011; 2012). These findings suggest that mechanical loading through exercise may have some bone-sparing effects under conditions of long-term energy deficiency, which we did not show in this short-term experiment.

Changes in regulatory hormones, indicative of energy conservation; namely reductions in IGF-1, leptin, T_3 and insulin, were shown following the low EA conditions; with these findings being in agreement with those of short-term energy deprivation experiments (Chapter 4; Ihle & Loucks, 2004; Friedl et al., 2000; Zanker & Swaine, 2000). Decreases in IGF-1 and leptin were shown independent of origin of energy restriction, whereas decrements in T_3 took place in diet-induced low EA only, and decreases in insulin in exercise-induced low EA only. When comparing diet- and exercise-induced low EAs, there

were no differences in regulatory hormones, which is in line with previous findings in a study that used the same level of low EA (15 kcal·kg $LBM^{-1}·d^{-1}$) (Koehler et al., 2016). These alterations in regulatory hormones occurred despite the absence of significant alterations in either bone formation or resorption, therefore, our findings do not support that these hormones mediate BTM responses, at least within the timeframe of our study.

No effects of either diet- or exercise induced low EA on 17*β*-oestradiol were shown, which supports the absence of significant change in bone formation and resorption in this investigation. These results are also in line with our 17*β*-oestradiol findings in Study 1 (Chapter 4) conducted in physically active women. In contrast, 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 $kcal·kgLBM^{-1}·d^{-1}$ (Ihle & Loucks, 2004). The discrepancies between the studies may in part be 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) (Ihle & Loucks, 2004). Other reproductive hormones, not determined in the current study, may also be negatively affected in response to EA. In the same series of experiments, LH pulsatility was suppressed at 10 and 20 $kcal·kgLBM^{-1}·d^{-1}$, with these findings suggesting that changes in gonadotrophins secreted by the anterior pituitary may precede changes in ovarian production of oestrogen in states of energy deficiency (Loucks & Thuma, 2003). As such, future studies should measure more reproductive hormones in relation to bone-related outcomes.

To conclude, 3 days of dietary-induced low EA implemented for 3 days resulted in a reduced BT ratio despite no significant effects of low EA on P1NP and *β*-CTX. The same level of low EA induced by exercise did not impact BTM reponses. Between diet- and exercise-induced low EAs there were no significant differences in BTM responses. However, our individual analysis revealed that more individuals experienced an increase in *β*-CTX, a decrease in P1NP or both in diet-induced low EA compared to exercise-induced low EA; suggesting a more consistent BTM response in diet-induced low EA compared to exercise-induced low EA. The present study suggests that imposing an energy deficit through diet or exercise does not affect bone formation or resorption within the 3-day timeframe of the present study, but this requires further investigation. There were no significant effects on BTM following reduced EAs despite a reduction in regulatory hormones. In future studies of longer duration, it would be interesting to investigate if differences in BTM responses to diet versus exercise induced reduced EA become evident.

Chapter 8. Bone metabolic response to short-term low energy availability achieved by diet or exercise individually in combined oral contraceptive users

8.1. Introduction

In the previous chapters (Chapter 4 and 7) the focus was placed upon physically active eumenorrheic women and their bone metabolism in response to low EA at 15 kcal·kgLBM^{-1}·d^{-1}. There is an increasing number of women using hormonal contraception for contraceptive and non-contraceptive purposes, with OCP and specifically combined OCP being the most common form prescribed by health care professionals (Lader et al., 2009). Given this trend in the general population, it is not surprising that the prevalence rates of OCP users among physically active women at least equals those of non-users (Bennell et al., 1999a; Burrows & Peters, 2007). The use of this type of contraception exceeds 50% in younger women (<30 years) (Lader, 2009) during an important time for bone mass acquisition and consolidation (Scholes et al., 2010; Rizzoli et al., 2010). Combined OCP users have down-regulated endogenous oestradiol levels due to the administration of exogenous oestrogens (Burrows & Peters, 2007). Thus, they can be used as an age-matched group to eumenorrheic women in order to compare consistently low levels of endogenous oestradiol to the cyclical effects of endogenous oestradiol (Elliott-Sale et al., 2013). Importantly, the low levels of oestradiol in combined OCP users are comparable to the levels of endogenous oestradiol of post-menopausal women (Heshmati et al., 2002), in whom accelerated bone turnover and bone loss are often observed (Feng & MacDonald, 2011; Burghardt et al., 2011; Riggs et al., 1998).

The studies and systematic reviews that have explored the effects of OCP use on BTM (Hermann & Seibel, 2010; Garnero et al., 1995; de Papp et al., 2007; Elgan et al., 2003) and BMD (Cibula et al., 2012; Liu & Lebrun, 2006; Recker et al., 1992; Pettiti et al., 2000; Reed et al., 2003) in the general population have yielded mixed results. Some have demonstrated increases or no change (Recker et al., 1992; Pettiti et al., 2000; Reed et al., 2003) in BMD, with others reporting decreases in BMD (Scholes et al., 2010; 2011; Cibula et al., 2012; Polatti et al.,1995). Similarly, BTM levels in OCP users have been reported to be lower (Garnero et al., 1995; de Papp et al., 2007) or similar to those of non-users (Elgan et al., 2003). Current literature on OCP use on BTMs and BMD is inconclusive and confounded mainly by the type and dose of OCPs and age, underpinning the need for future research in the area with rigorous experimental design and control of those parameters.

Physically active women who take OCP may be at greater risk for impaired bone health than their sedentary counterparts, as suggested by studies showing detrimental effects of OCP use when combined with exercise (Hartard et al., 1997; Burr et al., 2000; Weaver et al., 2001). It is, however, unknown if these effects are further exacerbated when exercise contributes to

the creation of an energy deficit. This is a particular unique challenge for physically active women who are commonly exposed to low EA attained by exercise training and/or dietary energy deficits. These practices have been associated with BMD reductions and the development of bone injuries, with these interrelationships being described under the Female Athlete Triad (Nattiv et al., 2007; De Souza et al., 2014a) and the RED-S models (Mountjoy et al., 2014; 2015) in physically active women. Physically active, combined OCP users are a neglected female population with similar (if not greater) risk to attain low EA than non-users (Thein-Nissenbaum et al., 2014). As such, given the potential for altered bone metabolism and bone loss in combined OCP users, it is important to explore the bone metabolism in response to low EA in physically active combined OCP users independently. Moreover, the responses in combined OCP users need to be characterised in relation to those observed in their eumenorrheic (non-users) counterparts (data from Chapter 7).

The proposed mechanisms for low EA-associated alterations in bone metabolism and health include alterations in regulatory and reproductive hormones (Ihle & Loucks, 2004; De Souza et al., 2014). Major anabolic hormones including insulin, leptin and T_3 are reduced during periods of energy deficiency in both physically active (Chapter 4) and sedentary women (Ihle & Loucks, 2004). Combined OCP use is often accompanied by hormonal changes including decreases in IGF-1 and increases in IGFBPs and T_3 levels (Blackmore et al., 2011; Wiegratz et al., 2003; Hansen et al., 2009). However, the effects of low EA on regulatory hormones in combined OCP users remain unknown. Oestrogen deficiency arising from low EA is one of the well-established mechanisms of bone loss in women with anorexia nervosa and exercise-induced amenorrhea (Misra, 2012). The impact of low EA on oestradiol levels in combined OCP users with an already downregulated oestradiol profile needs to be elucidated.

Comparing the effects of low EA in women with endogenous control and exogenous hormone regulation would be particularly useful given the increasingly prevalent OCP use, often over long time periods and from an early age, even before peak bone mass is achieved (Hartard et al., 2004; 2007). The aim of this study was to determine the effects of low EA achieved by diet or exercise on BTM in combined OCP users. A secondary goal was to compare these effects between combined OCP users and eumenorrheic (EU) women (data previously reported in Chapter 7). It was hypothesised that bone formation will decrease and bone resorption will increase following restricted EA in combined OCP users and these responses will be more severe than those in EU women.

8.2. Methods

8.2.1. Participants

Ten participants took part in the current study. Participants were included if they were taking a monophasic, low-dose monophasic Combined OCP (containing less than 50 mcg of ethinyl-oestradiol and a synthetic progestin) for at least 3 months prior to recruitment (Table 8.1) (Burkman et al., 2011). This type of combined OCP was chosen over others as it is the most common prescription of OCP (Lader et al., 2009). The rest of the inclusion and exclusion criteria were the same for EU women and combined OCP users and have been described in section 7.2.1.

Table 8.1. Combined OCP formulations taken by the participants

ID	Brand	Synthetic oestrogens	Synthetic progestins
003	Microgynon®	30 mcg ethinyl-oestradiol	150 mcg levonorgestrel
005	Rigevidon®	30 mcg ethinyl-oestradiol	150 mcg levonorgestrel
007	Microgynon®	30 mcg ethinyl-oestradiol	150 mcg levonorgestrel
009	Millinette®	30 mcg ethinyl-oestradiol	75 mcg gestodene
011	Gederal®	30 mcg ethinyl-oestradiol	150 mcg desogestrel
015	Yasmin®	30 mcg ethinyl-oestradiol	300 mcg drospirenone
018	Microgynon®	30 mcg ethinyl-oestradiol	150 mcg levonorgestrel
020	Yasmin®	30 mcg ethinyl-oestradiol	300 mcg drospirenone
021	Microgynon®	30 mcg ethinyl-oestradiol	150 mcg levonorgestrel
024	Lucette®	30 mcg ethinyl-oestradiol	300 mcg drospirenone

8.2.2. Experimental design

The experimental design has been described in 7.2.2. (Figure 7.1.).

8.2.3 Experimental procedures

All the procedures during the experimental period have been described previously in detail in section 7.2.3.

8.2.4. Blood samples

Blood samples were collected and analysed as previously described in 7.2.3.3.

8.2.5. Biochemical analysis

Detailed description on biochemical analysis has been provided in section 3.13.

8.2.6. Statistical analysis

Statistical analysis for combined OCP users has been described in section 7.2.6. For the comparison between EU women and combined OCP users, all data were checked for normality according to the Shapiro-Wilk test. Participant characteristics between EU women and combined OCP users were compared using independent t-tests for normally distributed data or Wilcoxon-rank sum tests for non-normally distributed data. Similarly, baseline biochemistry markers and markers of energy status prior to each experimental condition were averaged and compared using independent t-tests or Wilcoxon-rank sum tests for non-normally distributed data. Data were log-transformed before ANOVAs when not normally distributed. A three-way, mixed model, repeated measures ANOVA with group (EU, combined OCP) as a between subject factor and condition (CON, D-RES, E-RES) and time (BASE, D6) as within subject factors was used to assess group changes in BTMs, energy regulatory hormones and markers of calcium metabolism. A two-way, mixed model, repeated measures ANOVA was used to determine differences between EU women and combined OCP users over condition for BTM, energy regulatory hormones and markers of calcium metabolism expressed as AUC. Tukey's tests were used as *post hoc* analyses when a significant effect was found. Data are presented as mean (1SD) and statistical significance was set at $P \leq 0.05$. Data were analysed using Statistica 13.0 (Statsoft, USA). Criteria for responders and non-responders to D-RES and E-RES for β-CTX and P1NP have been established in section 7.2.7.

8.3. Results

The results for EU women have already been reported in Chapter 7. The results for OCP users are presented first and the direct comparison between OCP users and EU women follows for each outcome. The results for the combined data for combined OCP users and EU women are presented in Appendix 15.

8.3.1. Participant characteristics

Demographics, body composition and training characteristics for combined OCP users and EU women are presented in Table 8.2. There were no differences in any variable, which indicates that EU women and OCP users did not differ in terms of key characteristics.

Table 8.2. Participant characteristics

	Combined OCP (n=10)	**EU women (n=10)**	**p-value**
Demographics			
Age (y)	27 (4)	24 (3)	0.11
Height (m)	1.66 (0.04)	1.66 (0.06)	0.84
Body mass (kg)	58.1 (4.7)	61.1 (6.8)	0.28
BMI (kg ·m^2)	21.3 (1.4)	22.3 (2.4)	0.29
Body composition			
Body fat (%)	26.4 (3.9)	29.3 (5.1)	0.17
LBM (kg)	41.1 (3.3)	41.3 (4.1)	0.91
FFM (kg)	43.5 (3.4)	44.3 (4.3)	0.69
BMD (g·cm^2)	1.16 (0.07)	1.19 (0.09)	0.40
Training characteristics			
VO_2 peak (ml·kg^{-1}·min^{-1})	47.9 (5.5)	48.1 (3.3)	0.54
VO_2 peak (ml·kg LBM^{-1}·min^{-1})	69.9 (8.4)	70.9 (2.8)	0.73
Physical activity (MET-min·week^{-1})	3498 (1181)	4639 (2382)	0.19
Dietary and EE characteristics			
Habitual DEI (kcal·d^{-1})	2164 (377)[1]	1999 (371)[2]	0.38
Lifestyle EE (kcal·d^{-1})	382 (137)[2]	415 (119)[2]	0.57
24-hour EE(kg ·d^{-1})	1972 (167)[2]	2053 (198)[2]	0.67

Values are expressed as means (1SD).
[1]Analysis performed in 8 participants with completed data.
[2]Analysis performed in 9 participants with completed data.
[*] denotes a significant difference between combined OCP users and EU women (P<0.05).
BMI: Body Mass Index; BMD: Bone mineral density: VO_{2max}: Maximum oxygen uptake; DEI: Dietary Energy Intake; EE: Energy Expenditure; RMR: Resting Metabolic Rate; LBM: Lean Body Mass; FFM: Fat free mass; MET: Metabolic equivalents; OCP: Oral Contraceptive Pill Users; EU: Eumenorrheic

8.3.2. Baseline characteristics

Combined OCP users had significantly lower P1NP, Mg and 17*β*-oestradiol levels compared to EU women at BASE. (P<0.05). There were no other differences in any BTMs, marker of calcium metabolism or regulatory hormone (Table 8.3.).

Table 8.3. BASE concentrations of BTMs, markers of calcium metabolism and regulatory hormones in EU women and combined OCP users. Data are presented as the mean BASE values prior to CON, D-RES and E-RES.

	Combined OCP users (n=10)	**EU women (n=10)**	**P-value**
BTMs			
***β*-CTX (µg·L^{-1})**	0.40 (0.13)	0.48 (0.20)	0.31
P1NP (µg·L^{-1})	40.7 (13.1)	55.6 (13.9)	0.024*
BT ratio	1.05 (0.26)	1.23 (0.22)	0.12
Markers of calcium metabolism			
PTH (pg·mL^{-1})	3.5 (1.0)	4.3 (0.8)	0.057
ACa (mmol·L^{-1})	2.32 (0.08)	2.29 (0.07)	0.20
Mg (mmol·L^{-1})	0.79 (0.04)	0.82 (0.02)	0.047*
PO_4 (mmol·L^{-1})	1.29 (0.09)	1.27 (0.11)	0.59
Regulatory and reproductive hormones			
IGF-1 (mmol·L^{-1})	197.5 (54.8)	209.4 (52.4)	0.56
T_3 (mmol·L^{-1})	1.80 (0.31)	1.52 (0.30)	0.054
Leptin (ng·mL^{-1})	6.6 (3.7)	2.9 (1.6)	0.62
Insulin (pmol·L^{-1})	42.5 (18.0)	37.0 (14.8)	0.47
***17β*-oestradiol**	73.8 (51.5)	125.4 (42.4)	0.025*

Values are expressed as means (1SD).
* denotes a significant difference between COMBINED OCP users and EU women (P<0.05).
β-CTX: C-terminal telopeptides of type I collagen; P1N: Amino-terminal pro-peptide of Type 1 Procollagen; BT ratio: Bone turnover ratio; BTM: Bone turnover marker; 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; CON: Controlled; D-RES: Diet-induced restricted trial; E-RES: Exercise-induced restricted trial; OCP: Oral contraceptive pill; EU: Eumenorrheic.

8.3.3. BTMs

8.3.3.1. *β*-CTX

In combined OCP users, there was no significant main effect of time (P=0.31), condition (P=0.34) or any condition x time interaction effect (P=0.71) for *β*-CTX concentrations. *β*-CTX AUC did not significantly differ by condition (P=0.86) (Figure 8.1., Table 8.4).

When comparing between EU women and combined OCP users, there was no significant condition x time x group (P=0.42), condition x group (P=0.32) or any condition x time (P=0.63) interaction effect for β-CTX concentrations. There was no significant main effect of time (P=0.48), condition (P=0.10) or group (P=0.10). For β-CTX AUC, no significant main effect of condition (P=0.85), group (P=0.10) or any condition x group interaction effect (P=0.47) effect was shown (Figure 8.1., Table 8.4).

8.3.3.2. P1NP

In combined OCP users, there were no significant main effects of condition (P=0.08) or time (P=0.80), nor was there any condition x time interaction effect (P=0.97) for P1NP concentrations. P1NP AUC was not significantly different between CON, D-RES and E-RES (P=0.67) (Figure 8.1., Table 8.4). When comparing between EU women and combined OCP users, there was no significant condition x time x group (P=0.31), condition x group (P=0.71) or any condition x time (P=0.42) interaction effects for P1NP concentrations. There was a significant main effect of condition (E-RES<CON; P=0.046) and group (combined OCP users<eumenorrheic women; P=0.02), but no siginifcant effect of time (P=0.07). For P1NP AUC, no significant main effect of condition (P=0.78), group (P=0.13) or any condition x group interaction effect (P=0.25) effect was shown (Figure 8.1., Table 8.4).

8.3.3.3 BT ratio

In combined OCP users, no significant main effect of time (P=0.89) or any condition x time interaction effect (P=0.54) were shown for BT ratio but there was a significant effect of condition (P=0.048), although no *post hoc* pairwise comparisons reached statistical significance. BT ratio AUC did not significantly differ between CON, D-RES and E-RES (P=0.91) (Figure 8.1., Table 8.4).

When comparing between EU women and combined OCP users, there was no significant condition x time x group (P=0.14), condition x group (P=0.71) or condition x time (P=0.42) interaction effect for BT ratio. There was a significant main effect of time (D6<BASE; P=0.027) and condition (D-RES<CON, P=0.049; D-RES<E-RES, P=0.022), but no significant main effect of group (P=0.45). For BT ratio AUC, there was a main effect of group (EU<Combined OCP, P=0.020), but no main effect of condition (P=0.30) or any condition x group interaction (P=0.24) (Figure 8.1., Table 8.4).

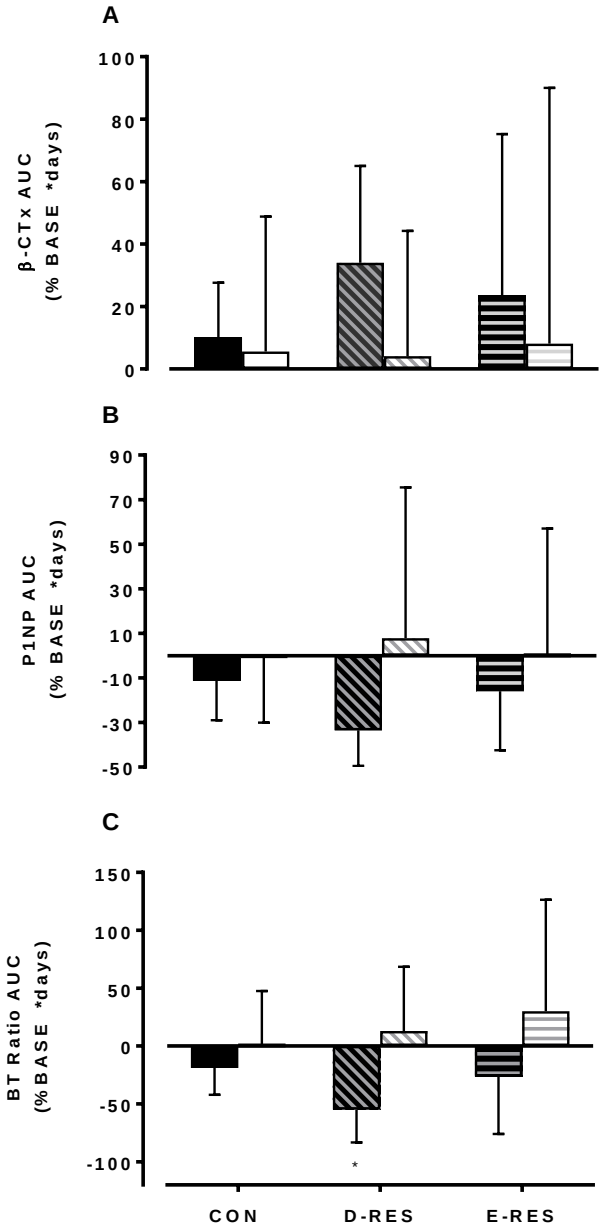


Figure 8.1. AUC analysis of β-CTX (A), P1NP (B) and BT ratio (C) in EU women (black) and combined OCP users (white) in CON (plain pattern), D-RES (diagonal hatched pattern) and E-RES (horizontal hatched pattern). Values are presented as mean (1SD). *denotes a significant difference from CON ($P<0.05$). *β*-CTX: C-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; D-RES: Diet-induced; E-RES: Exercise-induced restricted trial; BASE: Baseline; AUC: Area under the curve; OCP: Oral contraceptive pill; EU: Eumenorrheic.

Table 8.4. Markers of BTMs expressed as concentrations, percentage change from BASE and AUC in CON, D-RES and E-RES trials in combined OCP users and EU women. Values at D2 were used as BASE prior to each experimental condition.

	EU women (n=10)						Combined OCP users (n=10)					
	CON		D-RES		E-RES		CON		D-RES		E-RES	
	BASE	D6	BASE	D6	BASE	D6	BASE	D6	BASE	D6	BASE	D6
***β*-CTX**												
µg·L^{-1}	0.50	0.51	0.48	0.55	0.47	0.49	0.38	0.38	0.42	0.40	0.41	0.37
	(0.19)	(0.18)	(0.18)	(0.17)	(0.24)	(0.18)	(0.14)	(0.13)	(0.14)	(0.14)	(0.12)	(0.11)
%BASE change		5.0		17.0		11.7		2.8		-2.1		-4.1
		(8.8)		(15.5)		(25.8)		(21.6)		(20.1)		(41.0)
AUC		10.0		34.0		23.5		5.6		-4.1		-8.1
(%BASE x d)[1]		(17.7)		(31.0)		(51.8)		(43.2)		(40.1)		(81.9)
P1NP												
µg·L^{-1}	56.7	52.5	54.8	45.2	55.3	50.9	40.9	39.7	38.8	38.0	42.5	41.3
	(16.9)	(11.9)	(12.7)	(9.3)	(14.4)	(15.8)	(11.4)	(8.3)	(14.8)	(11.2)	(14.8)	(12.6)
%BASE change		-5.6		-16.8		-8.0		-0.5		3.9		0.0
		(8.9)		(8.0)		(13.3)		(14.5)		(33.8)		(28.3)
AUC		-11.1		-33.5		-16.0		-1.0		7.9		0.0
(%BASE x d) [1]		(17.8)		(16.0)		(26.5)		(29.0)		(67.6)		(56.7)
BT ratio												
-	1.19	1.06	1.21	0.85	1.30	1.06	1.15	1.09	0.94	0.95	1.06	1.14
	(0.24)	(0.20)	(0.26)	(0.14)	(0.39)	(0.23)	(0.35)	(0.19)	(0.27)	(0.17)	(0.28)	(0.24)
%BASE change		-9.5		-27.4		-13.1		0.5		6.5		15.0
		(12.0)		(14.2)		(24.9)		(23.2)		(27.7)		(48.1)
AUC		-19.0		-54.9[*]|		-26.2		1.0		13.1		30.0
(%BASE x d) [1]		(23.2)		(28.5)		(49.7)		(46.4)		(55.4)		(96.3)

Values are expressed as means (1SD). [1]AUC calculated for each experimental condition from BASE to D6. [*]denotes a significant difference from BASE in the same condition ($P<0.05$).
β-CTX: C-terminal cross-linked telopeptide of type I collagen; P1NP: Amino-terminal pro-peptide of type 1 procollagen; BT ratio: Bone turnover ratio; BTM: Bone turnover marker; CON: Controlled trial; D-RES: Diet-induced E-RES: exercise-induced restricted trial; BASE: Baseline; AUC: Area under the curve; OCP: Oral contraceptive pill users; EU: Eumenorreic.

8.3.4. Markers of calcium metabolism

8.3.4.1. PTH

In combined OCP users, there was no significant main effect of time (P=0.16), condition (P=0.29), or any condition x time interaction effect (P=0.33) for PTH concentrations. PTH AUC did not differ by condition (P=0.43) (Table 8.5.).

When comparing between EU women and combined OCP users, there was no significant condition x time x group (P=0.99), condition x group (P=0.24) or any condition x time (P=0.74) interaction effect for PTH concentrations. There were significant main effects of group (EU women>combined OCP users, P=0.008) and time (D6<BASE, P=0.02), but no significant main effect of condition (P=0.27). For PTH AUC, a significant main effect of group (combined OCP users <EU women, P<0.001) was shown, but there was no main condition effect (P=0.17) or any condition x group interaction effect (P=0.60) (Table 8.5.).

8.3.4.2. ACa

In combined OCP users, there was no significant effect of time (P=0.48) or condition (P=0.07). A condition x time interaction (P=0.03) was shown for ACa concentrations. Concentrations at D6 in D-RES were higher compared to BASE and concentrations at D6 in E-RES, with these findings being in line with the multi-mineral supplementation in D-RES but not E-RES. ACa AUC were significantly lower in CON (P=0.003) and E-RES (P=0.003) compared to D-RES (Table 8.5.).

When comparing between EU and combined OCP users, there was a significant condition x time x group interaction. In combined OCP users, ACa levels were higher at D6 from BASE in D-RES and were also higher from concentrations at D6 in E-RES. In EU women, levels at D6 were higher in CON compared to E-RES. There was also a condition x time interaction with concentrations at D6 in E-RES being lower than in D-RES (P=0.006) and CON (P=0.002). There was a main condition effect (D-RES<CON (P=0.019), but there were no significant main effects of time (P=0.82) or group (P=0.12). For ACa AUC, there was a significant main effect of condition (D-RES>E-RES, P=0.006) and a condition x group interaction effect (P=0.025) (Table 8.5.). In combined OCP users only, ACa AUC in D-RES was higher than CON (P=0.040) and E-RES (P=0.036) (Table 8.5.).

8.3.4.3. Mg

In combined OCP users, there was a significant effect of time (D6<BASE; P=0.028) but no significant main effect of condition (P=0.32) or any condition x time interaction effect (P=0.43) for Mg levels. Mg AUC did not significantly differ by condition (P=0.42) (Table 8.5.).

When comparing EU women and combined OCP users, there was no significant condition x time x group interaction (P=0.77), condition x group interaction (P=0.18) or any condition x time (P=0.65) interaction effect for Mg levels. There was no significant main effects of condition (P=0.99), time (P=0.15) or group (P=0.15) for Mg mean concentrations. Mg AUC was significantly lower in EU women than combined OCP users (group effect, P=0.031). There was no significant main effect of condition (P=0.61) or any condition x group interaction effect (P=0.78) (Table 8.5.).

8.3.4.4. PO_4

In combined OCP users, there was a significant main effect of time (D6<BASE; P=0.007) but no significant condition (P=0.48) or condition x time interaction (P=0.97) for PO_4 concentrations. PO_4 AUC did not significantly differ by condition (P=0.96) (Table 8.5.).

When comparing EU women and combined OCP users, there was no significant condition x time x group interaction (P=0.09) or any condition x group interaction (P=0.95). There was a condition x time interaction effect (P=0.043), with levels at D6 decreasing from BASE (P=0.001) in E-RES only. There was a significant main effect of time (P=0.002), but no significant main effects of condition (P=0.40) or group (P=0.15) for PO_4 mean concentrations. There was no main effect of group (P=0.080) or any condition x group interaction effect (P=0.09), but a significant main effect of condition (E-RES< D-RES, P=0.030) was shown for PO_4 AUC (Table 8.5.).

Table 8.5. Markers of calcium metabolism expressed as concentrations, percentage change from BASE and AUC in CON, D-RES and E-RES trials in combined OCP users and EU women. Values at D2 were used as BASE prior to each experimental condition.

	EU women (n=10)						Combined OCP users (n=10)					
	CON		D-RES		E-RES		CON		D-RES		E-RES	
	BASE	D6	BASE	D6	BASE	D6	BASE	D6	BASE	D6	BASE	D6
PTH												
pg·mL^{-1}	4.37	3.78	4.03	3.69	4.60	4.09	3.03	3.05	3.89	3.19	3.65	3.06
	(1.13)	(0.49)	(0.92)	(0.69)	(1.44)	(0.78)	(0.51)	(0.58)	(1.97)	(0.80)	(0.89)	(0.44)
%BASE change		-8.0		-4.3		-4.3		1.5 (18.7)		-6.8		-11.7
		(26.4)		(26.5)		(31.4)				(31.2)		(26.0)
AUC		-15.6		-8.5		-8.6		3.1 (37.4)		-13.7		-23.3
(%BASE x d)[1]		(52.8)		(52.9)		(62.8)				(62.3)		(52.0)
ACa												
mmol·L^{-1}	2.30	2.31	2.27	2.27	2.29	2.26	2.35	2.33	2.31	2.36	2.31	2.30
	(0.05)	(0.04)	(0.03)	(0.04)	(0.04)	(0.03) **	(0.09)	(0.9)	(0.08)	(0.09)*	(0.10)	(0.07)†
%BASE change		0.8		0.0		-1.4		-0.5		2.2		-0.5
		(2.1)		(2.1)		(1.4)		(1.7)		(2.0)		(2.5)
AUC		1.5		-0.7		-2.7		-1.0		4.3		-1.0
(%BASE x d) [1]		(4.2)		(4.2)		(2.8)		(3.3)†		(3.9)		(5.1)†
Mg												
mmol·L^{-1}	0.83	0.82	0.81	0.81	0.81	0.82	0.79	0.79	0.79	0.81	0.79	0.81
	(0.02)	(0.03)	(0.03)	(0.06)	(0.03)	(0.04)	(0.05)	(0.06)	(0.04)	(0.04)	(0.06)	(0.05)
%BASE change		-0.7		-0.7		0.4		0.4		2.7		2.4
		(2.3)		(7.0)		(5.4)		(3.4)		(2.1)		(5.7)
AUC		-1.5		-1.4		0.9		0.7		5.3		4.7
(%BASE x d) [1]		(4.6)		(14.0)		(10.7)		(6.7)		(4.3)		(11.4)

Markers of calcium metabolism (continue)	**EU women (n=10)**						**Combined OCP users (n=10)**					
	CON		**D-RES**		**E-RES**		**CON**		**D-RES**		**E-RES**	
PO_4	**BASE**	**D6**	**BASE**	**D6**	**BASE**	**D6**	**BASE**	**D6**	**BASE**	**D6**	**BASE**	**D6**
$mmol \cdot L^{-1}$	1.29	1.28	1.26	1.31	1.33	1.20	1.27	1.18	1.28	1.19	1.25	1.15
	(0.12)	(0.12)	(0.14)	(0.08)	(0.15)	(0.10)	(0.12)	(0.13)	(0.12)	(0.12)	(0.09)	(0.07)
%BASE change		-0.5		4.6		-9.4		-6.8		-6.6		-7.7
		(8.6)		(11.0)		(10.3)		(10.8)		(9.4)		(8.2)
AUC		-0.9		9.3		-18.8		-13.7		-13.2		-15.5
(%BASE x d) [1]		(17.2)		(21.9)		(20.5)		(21.6)		(18.7)		(16.4)

Values are expressed as means (1SD).

[1]AUC calculated for each experimental condition from BASE to D6.

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

[**] denotes a significant difference from D-RES at the same time point (P<0.05).

[†]denotes a significant difference from D-RES at the same time point (P<0.05).

PTH: Parathyroid hormone; Mg: Magnesium; ACa: Albumin adjusted Calcium; PO_4: Phosphate; D;Day; BASE: Baseline; AUC: Area under the curve; CON, Controlled trial; D-RES, Diet-induced restricted trial; E-Exercise-induced restricted trial; OCP: Oral contraceptive pill; EU: Eumenorrheic.

8.3.6. Regulatory hormones

8.3.6.1. IGF-1

In combined OCP users, IGF-1 concentrations were significantly reduced at D6 compared to BASE (main effect of time; P=0.010). IGF-1 concentrations were lower in E-RES compared to CON (P=0.006) and D-RES (P=0.002). There were, however, no significant differences between condition for any time point for IGF-1 concentrations (condition x time interaction effect, P=0.23). IGF-1 AUC was not significantly different across experimental conditions (P=0.07) (Table 8.6.).

When comparing between EU women and combined OCP users, there was a significant condition x time x group interaction effect (P=0.001). In both combined OCP users and EU women, concentrations at D6 in E-RES decreased from BASE (P values 0.02 and <0.001) and were lower than concentrations at D6 in CON (P values 0.02 and <0.001). Concentrations at D6 in D-RES decreased from BASE by 12.2% (P=0.02) in combined OCP users and by 13.2% (P=0.008) in EU women. Concentrations at D6 in D-RES were different from D6 levels in CON (P<0.001) in EU women only. There was a significant condition x group (P=0.02) interaction effect, with lower levels of IGF-1 in D-RES (P=0.015) and E-RES (P=0.04) compared to CON in EU women. There was a significant condition x time interaction effect for IGF-1 concentrations (P<0.001), IGF-1 concentrations at D6 decreased from BASE in D-RES (P<0.001) and E-RES (P<0.001). IGF-1 concentrations at D6 in D-RES and E-RES were lower than concentrations at D6 in CON (P<0.001). IGF-1 concentrations at D6 in D-RES were significantly lower than those in E-RES (P=0.003). There was a significant main effect of time (P<0.001) and condition (E-RES<CON, P=0.001), but there was no significant main effect of group (P=0.51). The between combined OCP users and EU women comparison, showed a significant main condition (D-RES<CON; P<0.001, E-RES<CON; P<0.001) for IGF-1 AUC, but no significant main effect of group (P=0.89). In EU women only, IGF-1 AUC in D-RES (P<0.001) and E-RES (P<0.001) were lower than CON (condition x group, P=0.002) (Table 8.6).

8.3.6.2. T_3

In combined OCP users there was no significant main effect of time (P=0.67) or condition (P=0.09); but there was a condition x time interaction effect (P=0.005). T_3 concentrations were reduced at D6 from BASE in D-RES (P=0.028) and were lower than concentrations at D6 in CON (P=0.005) and E-RES (P=0.014). There was a significant main effect of

condition for T_3 AUC (P=0.005); AUC in D-RES was lower than in CON (P=0.01) and E-RES (P=0.01) (Table 8.6.).

When comparing between EU women and combined OCP users, no significant condition x time x group (P=0.40) or any condition x group (P=0.79) interaction effect was shown. There was, however, a condition x time interaction for T_3 concentrations (P<0.001); T_3 concentrations were reduced at D6 from BASE in D-RES (P<0.001) and were also lower than concentrations at D6 in CON (P<0.001) and E-RES (P=0.002). There were significant main effects of group (combined OCP users>EU, P=0.02), time (D6<BASE, P=0.026) and condition (D-RES<CON, P=0.012). The between EU women and combined OCP users comparison showed no significant main effect of group (P=0.17) or any condition x group interaction (P=0.48) effect for T_3 AUC. A significant main effect of condition was shown for T_3 AUC (P<0.001) showing that T_3 responses were lower in D-RES compared to CON (P<0.001) and E-RES (P=0.004) (Table 8.6).

8.3.6.3. Leptin

In combined OCP users, there was a significant effect of time (D6<BASE; P=0.010) and condition (E-RES<CON; P<0.001, D-RES< CON; P<0.001) and a condition x time interaction (P=0.23) effect for leptin concentrations. Leptin concentrations at D6 were reduced by 60.6% and 46.4% compared to BASE (P<0.001) in D-RES and E-RES respectively and were lower than D6 in CON (P<0.001). Leptin AUCs in D-RES (P<0.001) and E-RES (P=0.020) were lower than that in CON, but there was no significant difference between D-RES and E-RES (P=0.22) (Table 8.6.).

When comparing between EU women and combined OCP users, there was no significant condition x time x group (P=0.35) or condition x group (P=0.81) interaction effects for leptin concentrations. There was, however, a condition x time interaction for leptin concentrations (P<0.001); concentrations at D6 decreased from BASE in CON (P<0.001), D-RES (P<0.001) and E-RES (P<0.001). Concentrations at D6 in D-RES (P<0.001) and E-RES (P<0.001) were also lower than concentrations at D6 in CON. There were main effects of time (D6<BASE, P<0.001) and condition (D-RES<CON, P<0.001; E-RES<CON, P<0.001), but not a significant main effect of group (P=0.800). The comparison between EU women and combined OCP users showed no significant main effect of group (P=0.29) or any group x condition interaction (P=0.35) effect for leptin AUC. A main effect of condition was shown for leptin AUC (P<0.001) showing that leptin responses in D-RES (P<0.001) and E-RES (P<0.001) were lower than those in CON (Table 8.6).

8.3.6.4. Insulin

In combined OCP users, there was a significant main effect of time (D6<BASE; P=0.026). There was no main effect of condition (P=0.12) or any condition x time interaction (P=0.83) effect. Insulin AUC did not significantly differ by condition (P=0.69) (Table 8.6.).

When comparing between EU women and combined OCP users, no significant condition x time x group (P=0.18) or condition x group (P=0.74) interaction effects were shown. There was a condition x time interaction for insulin concentrations (P=0.045); concentrations at D6 were reduced from BASE in E-RES (P=0.001) and were lower than concentrations in CON at D6 (P=0.015). There was a main effect of time (D6<BASE, P<0.001), but no significant main effect of condition (P=0.053) or group (P=0.48). There was no significant main effect of condition (P=0.10), group (P=0.61) or any condition x group (P=0.44) interaction effect for insulin AUC (Table 8.6).

8.3.7. Reproductive hormones

Concentrations of 17β-oestradiol were below the analytical detections levels in 5 out of 10 combined OCP users for at least one-time point. There was a main effect of time for 17β-oestradiol levels (P=0.001) with lower levels at D6 compared to BASE. No significant main effect of condition (P=0.90) or condition x time interaction (P=0.94) effect were shown for 17β-oestradiol levels. AUCs for 17β-oestradiol did not significantly differ between conditions (P=0.41) (Table 8.6.).

When comparing between EU women and combined OCP users, there was only a main effect of group (combined OCP users< EU women, P<0.001) and a time x group interaction (P<0.001) for 17β-oestradiol concentrations. Concentrations were lower in combined OCP users compared to EU women at BASE (P=0.002) and D6 (P<0.001). In combined OCP users, 17β-oestradiol concentrations were reduced at D6 from BASE (P<0.001), but were not different between BASE-D6 in EU women (P=0.40) (Table 8.6.).

Table 8.6. Regulatory and reproductive hormones expressed as concentrations, percentage change from BASE and AUC in CON, D-RES and E-RES trials in combined OCP users and EU women. Values at D2 were used as BASE prior to each experimental condition.

	EU women (n=10)						**Combined OCP users (n=10)**					
	CON		**D-RES**		**E-RES**		**CON**		**D-RES**		**E-RES**	
	BASE	**D6**	**BASE**	**D6**	**BASE**	**D6**	**BASE**	**D6**	**BASE**	**D6**	**BASE**	**D6**
T_3												
mmol·L^{-1}	1.49 (0.34)	1.47 (0.24)	1.53 (0.28)	0.1.29 (0.17)	1.53 (0.31)	1.40 (0.21)	1.80 (0.30)	1.87 (0.41)	1.82 (0.34)	1.61 (0.37)*,**,†	1.77 (0.31)	1.84 (0.44)
%BASE change		0.0 (11.3)		15.0 (11.2)		6.8 (11.0)		3.7 (16.7)		-11.7 (8.8)		3.4 (13.2)
AUC (%BASE x d) [1]		0.5 (22.6)		-29.5 (22.4)		-13.7 (22.0)		7.4 (33.4)		-23.4 (17.6)**,†		6.9 (26.5)
IGF-1												
mmol·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)*,**	196.5 (47.9)†	185.3 (37.9)	207.1 (49.8)	180.9 (39.5)*	188.8 (51.5)	161.7 (33.4)*,**
%BASE change		10.6 (18.9)		-13.2 (9.1)		-23.4 (9.5)		-4.8 (8.4)		-12.2 (8.5)		-10.9 (21.4)
AUC (%BASE x d) [1]		21.3 (37.9)†		-26.4 (18.2)**		-46.7 (19.1)**		-9.5 (16.9)		-24.4 (16.9)		-21.8 (42.7)
Leptin												
ng·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)	6.7 (4.1)	5.1 (2.7)	7.1 (4.1)	2.7 (1.7)*,**	6.0 (3.3)	3.4 (2.6)*,**
%BASE change		-30.0 (22.7)		-59.0 (19.6)		-60.6 (16.7)		-21.9 (19.5)		-60.6 (13.7)		-46.4 (25.8)
AUC (%BASE x d) [1]		-60.0 (45.4)		-118.0 (39.1)		-121.3 (33.4)		-43.9 (39.0)		-121.2 (27.4)**		-92.8 (51.5)**

Regulatory and reproductive hormones (Continue)	EU women (n=10)						Combined OCP users (n=10)					
	CON		D-RES		E-RES		CON		D-RES		E-RES	
	BASE	D6	BASE	D6	BASE	D6	BASE	D6	BASE	D6	BASE	D6
Insulin												
pmol·L^{-1}	31.6	36.7	33.8	28.6	45.6	20.4	50.1	36.4	37.9	27.9	39.4	26.0
	(7.7)	(20.9)	(9.0)	(15.0)	(34.8)	(11.4)	(41.8)	(17.0)	(16.4)	(12.5)	(19.8)	(8.3)
%BASE change		13.0		-6.4		-36.5		-8.7		-19.9		-22.6
		(44.0)		(62.5)		(49.3)		(52.0)		(38.0)		(37.8)
AUC		26.0		-12.8		-73.0		-17.3		-39.9		-45.1
(%BASE x d)[1]		(87.9)		(125.0)		(98.5)		(104.0)		(76.1)		(75.7)
17-*β* oestradiol												
pmol·L^{-1}	108.9	157.3	118.9	157.9	148.3	167.0	68.8	27.5	71.9	29.1	80.7	29.0
	(33.6)	(53.1)	(29.7)	(62.9)	(92.9)	(72.1)	(63.3)	(9.9)	(52.8)	(11.4)	(89.8)	(13.3)
%BASE change		48.3		32.2		24.0		42.1		-41.0		-28.0
		(35.2)		(39.2)		(39.9		(25.9)		(34.8)		(42.2)
AUC		96.6		64.5		48.1		-84.3		-82.0		-56.0
(%BASE x d)[1]		(70.4)		(78.4)[	]	(79.8)		(51.9)		(69.5)		(84.3)

Values are expressed as means (1SD)

[1]AUC calculated for each experimental condition from BASE to Day 6.

[*]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).

[†]denotes a significant difference from E-RES at the same time point (P<0.05).

T_3: Triiodothyronine; IGF-1: Insulin-like growth factor 1; D: Day; BASE: Baseline; AUC: Area under the curve; CON: Controlled trial; D-RES, Diet-induced restricted trial; E-RES: Exercise-induced restricted trial; OCP: Oral contraceptive pill; EU: Eumenorrheic.

8.3.8. Individual analysis

Individual responses for *β*-CTX and P1NP and altered bone turnover due to increased β-CTX, decreased P1NP or both, in D-RES and E-RES trials, for combined OCP users and EU women are presented in Table 8.7.

Table 8.7. Number of responders (out of total number of EU women and COMBINED OCP users) for *β*-CTX, P1NP in EU women and combined OCP users in D-RES and E-RES. This analysis was based on data expressed as %BASE for each participant.

	***β*-CTX**		**P1NP**		**Bone turnover[1]**		**Bone turnover[2]**	
	D-RES	**E-RES**	**D-RES**	**E-RES**	**D-RES**	**E-RES**	**D-RES**	**E-RES**
Combined OCP users	2/10	1/10	3/10	1/10	4/10	3/10	1/10	0/10
EU women	5/10	4/10	5/10	3/10	8/10	5/10	2/10	2/10
Total	7/20	5/20	8/20	4/20	12/20	8/20	3/20	2/20

[1]altered bone turnover due to increased *β*-CTX, decreased P1NP or both.
[2]altered bone turnover due to a simultaneous increase *β*-CTX and decrease in P1NP.
β-CTX: C-terminal telopeptides of type I collagen; P1NP: Amino-terminal Pro-peptides of Type 1 Procollagen; BT ratio; BASE: Baseline; AUC: Area under the curve; CON, Controlled trial; D-RES, Diet-induced restricted trial; E-RES: Exercise-induced restricted trial; OCP: Oral contraceptive pill; EU: Eumenorrheic

8.4. Discussion

The current study showed that neither bone formation nor resorption were affected by 3 days of low EA in combined OCP users and that these effects were not different depending on whether reduced EA was diet- or exercise-induced. Direct comparison between combined OCP users and EU women showed no significant differences in BTM responses to either diet- or exercise-induced low EA. These results suggest that combined OCP use does not impact bone metabolism following short-term reduced EA. With respect to eumenorheic women, combined OCP users exhibit similar BTM responses when exposed to the same dietary and exercise conditions.

We hypothesised that our combined OCP users would have suppressed BTM levels at baseline. Indeed, there were significantly lower baseline levels of P1NP in COC users than EU women, although they were within the reference range (P1NP: 25-90 $\mu g \cdot L^{-1}$; Jenkins et al., 2013), which is in line with existing literature (de Papp et al., 2007). Baseline β-CTX concentrations were similar between the two groups, which is in agreement with some (Hansen et al., 2009), but not all previous studies (Hermann & Hermann, 2004). Given that combined OCP users and EU women did not differ in terms of key characteristics including demographics, body composition, training status, dietary and EE characteristics (please also see section 8.3.1. and 8.3.2.), we speculate that the differences seen in P1NP levels at baseline may be in part mediated by differences in oestradiol levels between the two groups. Oestradiol levels at baseline were significantly lower in combined OCP users compared to those in EU women, confirming former combined OCP studies showing a downregulation of endogenous oestradiol production through negative feedback provided to GnRH, LH and FSH during the exogenous provision of oestradiol and synthetic progestin (Burrows & Peters, 2007).

We did not show any effect on markers of bone formation, resorption or on the BT ratio following either a diet- or exercise induced low EA in the combined OCP group, despite an overall significant reduction in oestradiol levels (D6< BASE). BTMs provide information about global bone turnover but do not allow the characterisation of altered bone turnover at the local level (Lombardi et al., 2012a). As such, even though we did not show any change in BTMs, we cannot exclude alterations in combined OCP users in response to low EA at all skeletal sites (Cobb et al., 2007). The findings of the current investigation are in line with our 3-day study performed in eumeorrheic women (please refer to Chapter 7). By using slightly longer experimental protocols (4 or 5 days), previous studies have demonstrated

decrements in bone formation and increases in bone resorption following short-term reduced EA, achieved through diet and exercise (Chapter 4; Ihle & Loucks, 2004) or acute fasting (Grinspoon et al., 1995) in eumenorrheic women. However, the effects of low EA (or energy restriction) attained through any modality (diet and/or exercise) on BTMs in combined OCP users have not been previously addressed. This study is the first to demonstrate no negative influence on BTMs within 3 days of diet or exercise-induced low EA in physically active combined OCP users. As such, this study adds novel evidence to the database of the Female Athlete Triad (Nattiv et al., 2007; DeSouza et al., 2014a) and the RED-S models (Mountjoy et al., 2014) about a relevant, overlooked subset of women facing unique challenges regarding their bone health.

Diet and/or exercise-driven energy deficit has been reported to negatively alter bone metabolism (Villareal et al., 2008; Shah et al., 2011) and result in lower BMD in some (Gozansky et al., 2005; Chao et al., 2000; Shah et al., 2011), but not all (Ryan et al., 1998; Svenden et al., 1993) studies in post-menopausal women (or mixed populations of postmenopausal women and older men), who may have a comparable profile of reproductive hormones with combined OCP users (Heshmati et al., 2002). Differences in key methodological aspects do not allow direct comparisons between these findings in postmenopausal women and our results in combined OCP users. For example, exercise training in our study was specifically designed for young, healthy, lean, physically active women and was, therefore, considerably different in duration, intensity and frequency from those programmes targeting older individuals including postmenopausal women (Villareal et al., 2008; 2011); with these differences suggesting greater osteogenic stimuli in our intervention (Lanyon et al., 1996). Conversely, the narrow young age range of our lean and physically active combined OCP users and the strict criteria applied to secure the inclusion of healthy individuals provide novel information about the effects of energy deficiency in populations with downregulated oestradiol production and go beyond the age-related (often accompanied by secondary comorbidities) decline in bone metabolism and health (Feng & MacDonald, 2011; Burghardt et al., 2011; Riggs et al., 1998).

In both animal and human studies, reductions in oestrogen levels have been associated with decreases in ER-α levels on bone (Lanyon et al., 1996; 2004), reduced osteoblast sensitivity to mechanical loading (Armstong et al., 2007) and supressed bone formation (Lee et al., 2004), all potentially contributing to bone loss. Although the actions of progesterone on bone are less researched than those of oestrogens, these may be exerted directly on bone or imdirectly through androgens, corticosteroids and oestrogens, with these effects being influenced by the dose and type of progesterone administered (Nappi et al., 2012). In the

present study, all participants were taking combined OCP with the same dosage of ethinyl-oestradiol minimising the variation in this component but with different types and doses of progestins. Notably, we showed substantial inter-individual variability in circulating 17β-oestradiol (Liu & Lebrun, 2005; Bennell et al., 1999a). We did not measure progesterone levels, and are, therefore, unable to conclude whether or not alterations of this hormone have contributed to the observed variability in 17β- oestradiol and bone-related outcomes. These unexpected findings highlight the complex interactions between combined OCP and endogenous reproductive hormones, which warrant further investigation in future research.

In addition, combined OCP use has been shown to modify the concentrations of hormones associated with bone metabolism including testosterone and IGF-1 (decreases), IGFBPs (increase) and T_3 (increase) (Blackmore et al., 2011; Wiegratz et al., 2003; Hansen et al., 2009). In our study, reduced leptin, T_3, IGF-1 levels in response to low EA were shown, which are typical compensatory responses to energy deprivation (Chaper 4; De Souza et al., 2008; Ihle & Loucks 2004). However, these alterations were not supported by the BTM responses, suggesting that they were insufficient to suppress bone turnover at least within the 3-day experimental period.

We did not show any further differences between combined OCP users and EU women in BTM responses to either diet- or exercise-induced low EAs, despite significant differences in oestradiol levels at baseline (combined OCP< EU) and distinct alterations in oestradiol patterns between the groups throughout the 3-day exeperimental period. Mean oestradiol levels remained unchanged throughout BASE-D6 in EU women, but decreased from BASE at D6 in combined OCP users, although these changes did not occur as a result of low EA, at least within the short-term timeframe of the current study. These results may indicate that combined OCPs, containing 30 μg ethinyl-oestradiol, provide a sufficient stimulus for bone sparing effects (Horsman et al., 1983; Liu & Lebrun, 2005), even under conditions of low EA. Future studies need to address if these similarities in BTM response to low EA between combined OCP users and EU women persist over more prolonged periods of low EA, or if the selection of a different combined OCP (*i.e.*, <30 μg ethinyl-oestradiol) results in different BTM responses to low EA than those experienced by EU women.

Despite no differences between the two groups following either diet- or exercise-induced low EAs, the direction and mean magnitude of BTM responses were different between combined OCP users and eumenorrheic women. In the diet-induced low EA trial, β-CTX, P1NP and BT ratio were altered by +17%, -17% and -27% from BASE in eumenorrheic women, whereas, in combined OCP users, the respective changes were -2%, +4% and

+6.5%. Similarly, in the exercise-induced low EA trial, β-CTX increased by 12%, P1NP decreased by 8% and BT ratio was reduced by 13% in eumenorrheic women, whereas, in combined OCP users, β-CTX decreased by 4%, P1NP remained unaltered and BT ratio increased by 15%. Individual analysis showed that more EU women had altered bone metabolism due to increased β-CTX, decreased P1NP or both in diet-induced low EA (eumenorrheic women: 8/10 and combined OCP users: 4/10) and exercise-induced low EA (eumenorrheic women: 4/10 and combined OCP users: 3/10).

When the responses of regulatory hormones to low EA in eumenorrheic women and combined OCP users were compared directly, there were no significant differences between combined OCP users and eumenorrheic women. Overall, IGF-1 and leptin concentrations were reduced following reduced EA and T_3 was reduced in response to diet-induced EA only; with these findings being in agreement with similar short-term experiments (Chapter 4; Ihle & Loucks, 2004). Insulin decreased in the exercise-induced condition only, which is posiibly due to glycogen depletion and the resulting hypoglycaemia occurring in prolonged exercise bouts (Viru et al., 1992). Unlike previous short-term experiments reporting that similar hormonal alterations were associated with changes in BTMs (Ihle & Loucks, 2004; Zanker & Swaine, 2000), the current study does not support BTM changes mediated by these hormones within the 3-day timeframe.

When low EA is attained through dietary restriction, it is likely that reduced availability of specific nutrients may contribute to alterations in bone turnover (Shapses & Sukumar, 2012). Thus, we provided a multi-mineral, multi-vitamin supplement in this condition, but we did not provide one when reduced EA was attained through exercise. In line with the multi-mineral supplementation in diet-induced low EA trial, but not the exercise-induced low EA trial, we showed higher ACa in the diet-induced low EA trial than the exercise-induced low EA trial. There were no further effects of either reduced EA on PTH, Mg or PO_4 levels, supporting the absence of alterations in BTMs.

To conclude, there were no detrimental effects of combined OCP use on bone metabolism in response to either diet- or exercise- induced low EA. We also provided a comparative approach between eumenorrheic women and combined OCP users, showing no differences in BTM responses to low EAs between the groups. These findings are of clinical significance, supporting no negative effects of combined OCP use on bone metabolism following low EA in the short-term. As such, the current investigation adds novel evidence relevant to an increasing, but under-studied sub-population of the Female Athlete Triad and the RED-S models. Additional research on the longer-term impact of similar exercise and

dietary interventions on bone-related outcomes, namely BTM, BMD and bone quality, in combined OCP users is warranted.

Chapter 9. General discussion

Low EA is one of the main underlying factors associated with poor bone health, as identified by the Female Athlete Triad (Nattiv et al., 2007; De Souza et al., 2014) and the RED-S models (Mountjoy et al., 2014; 2015). To date, experimental studies on the impact of low EA on bone metabolism and health in relevant populations of physically active individuals are lacking. The overall aim of the current programme was to explore the short-term effects of low EA on BTM, regulatory and reproductive hormones and markers of calcium metabolism, which may mediate potential BTM effects in physically active individuals. This was accomplished through the implementation of four laboratory studies as part of the overall design of the programme. Studies 1 and 2 explored the effects of short-term low EA achieved by diet and exercise on BTMs in physically active, eumenorrheic women (reported in Chapter 4) and men (reported in Chapter 5). The data collected in Study 1 and 2 were combined to provide a direct comparison of BTM responses to short-term low EA between physically active women and men (reported in Chapter 6). Studies 3 and 4 investigated the effects of short-term low EA on BTM dependent on the way low EA was implemented, diet- or exercise-induced, in physically active, eumenorrheic women (reported in Chapter 7) and women using combined OCPs (reported in Chapter 8). The data collected in Studies 3 and 4 were directly compared to provide differences in BTM, regulatory and reproductive hormones and markers of calcium metabolism between these groups (reported in Chapter 8).

9.1. Overview of key findings

9.1.1. Bone metabolic response to short-term low EA achieved through diet and exercise in physically active, eumenorrheic women

In Study 1 (reported in Chapter 4), short-term low EA at 15 kcal·kgLBM^{-1}·d^{-1}, attained by a combination of dietary restriction and exercise, resulted in a significant increase in bone resorption (as evidenced by significantly higher β-CTX AUC) and a decrease in bone formation (as evidenced by significantly lower P1NP AUC). In the only previous experimental study using the concept of low EA, Ihle & Loucks (2004) demonstrated decreased bone formation at a threshold EA of <30 kcal·kg LBM^{-1}·d^{-1}, whereas bone resorption was increased at more severely reduced EA at 10 kcal·kg LBM^{-1}·d^{-1}. Bone metabolic activities, were measured by urinary NTX (bone resorption) and P1CP (bone formation), which are limited by analytical variability (please see section 2.5.4.) and thus, are not included in recent recommendations about reference BTMs, published by expert scientific bodies in the area of bone health and disease (please see section 2.5.5.) (Vasikaran et al., 2011; Bauer et al., 2012).

The findings from Ihle and Loucks (2004) have been used to support recommendations regarding the prevention and treatment of the Female Athlete Triad (De Souza et al., 2014a). These data were, however, from sedentary women who differ from their physically active counterparts in baseline BTM levels, body composition, training and bone adaptations (Nishiyama et al., 1988; Fallon et al., 2001; Mazzetti et al., 2011). The absence of any subsequent study (since 2004) to confirm these findings is surprising, especially given the growing body of evidence about the Female Athlete Triad following the ACSM position statement in 2007 (Nattiv et al., 2007). By extending these findings in sedentary women to encompass a representative population of physically active women, and by using reference standards BTMs (β-CTX and P1NP) (Vasikaran et al., 2011), the current study in physically active women is the first to demonstrate that low EA though diet and exercise may induce bone resorption that is not matched with a concomittent increase in bone formation (Figure 9.1.).

Changes in regulatory hormones including insulin, leptin and T_3 accompanied the BTM responses in the present study (Study 1-reported in Chapter 4). Such alterations in regulatory hormones may have occurred in an attempt to spare the available energy for essential bodily processes and may indicate potential initial adaptations to a state of energy deficiency (Loucks & Thuma, 2003; Ihle & Loucks, 2004). The actions of these hormones on osteoblast and osteoclast activity have been previously described in the literature and have been detailed throughout this thesis (please see sections 2.11. and 4.4.). We did not show any changes in 17β-oestradiol concentrations, possibly due to our short-term intervention and single measurement of 17β-oestradiol levels. Similarly, there were no effects of low EA on PTH, ACa, Mg or PO_4 levels, which are in line with the provision of a multi-mineral supplement during low EA.

9.1.2. Bone metabolic response to short-term low EA achieved through diet and exercise in physically active men

Study 2 (reported in Chapter 5) explored the effects of low EA achieved through diet and exercise on bone metabolic response in physically active men. This study showed that low EA at 15 kcal·kgLBM^{-1}·d^{-1} had no effect on BTMs in physically active men. IGF-1 was significantly reduced following low EA, but no other regulatory hormones including T_3, insulin and leptin or markers of calcium metabolism were changed. Individual differences in susceptibility and responsiveness of BTM to low EA might suggest that, despite the overall BTM responses, some men are susceptible to low EA. Importantly, the prescription of low EA in the current study (reported in Chapter 5) was based upon previous studies showing

negative health outcomes in women (Thong et al., 2000) and thus, low EA was characterised at 15 kcal·kgLBM^{-1}·d^{-1}, similar to some (Koehler et al., 2016), but not all previous studies (<30 kcal·kgLBM^{-1}·d^{-1}; Viner et al., 2015). The current study (reported in Chapter 5) shows that BTMs are not altered due to low EA at 15 kcal·kgLBM^{-1}·d^{-1} underpining the need for future research to determine meaningful ranges and cut-offs for optimal and low EA related to clinical outcomes in men.

Studies 1 and 2 (reported in Chapters 4 and 5) were conducted under strict experimental conditions with numerous control measures. These include strict inclusion and exclusion criteria to eliminate confounding factors, careful consideration of blood sampling (baseline blood samples were collected prior to each experimental condition, after an overnight fast and at several time-points over the experimental protocol) and selection of BTMs of reference standards (Vasikaran et al., 2011). These critical aspects of our experimental design strengthen our approach to explore the effects of low EA on bone metabolism in physically active women and men.

9.1.3. A sex comparison: Bone metabolic response to short-term low EA in physically active women and men

The data from Study 1 (women-reported in Chapter 4) and Study 2 (men- reported in Chapter 5) were combined to directly compare BTMs, regulatory hormones or markers of calcium metabolism responses to low EA at 15 kcal·kgLBM^{-1}·d^{-1} between physically active women and men (Figure 9.1.). The main findings of this analysis were that there were no significant differences in any of these outcome measures between sexes; with the magnitude of the changes in BTM to short-term low EA achieved by exercise and dietary energy restriction being similar in women and men. This combined analysis adds novelty in the research area of bone turnover and low EA and offers a number of strengths including the prescription of the same level of low EA, expression of energy restriction relative to LBM and same contributions of dietary restriction and exercise for both sexes. Additionally, men and women had similar characteristics including age, training status (VO_2 peak relative to LBM rather than body mass) and lifestyle physical activity levels, but not in terms of body composition (LBM, % body fat) in order to retain ecological validity in line with real-world sex differences in these measurements. It should be noted, however, that the studies were conducted independently and, as such, the analysis was originally powered to detect differences in BTMs due to low EA in men and women independently. As such, future studies should confirm these findings in studies designed to detect sex differences.

The analysis of combined men and women data (provided by the ANOVA model) has some advantages, which include the generation of a larger sample size and the ability to determine an overall relationship between EA and bone metabolism in a mixed population of physically active individuals. We showed a significant reduction in P1NP and BT ratio, but not in β-CTX in response to low EA, findings that are in agreement with existing literature suggesting that bone formation is affected first under conditions of energy deficiency (Ilhe & Loucks, 2004; Zanker & Swaine 2000). The changes in BTMs in RES were accompanied by alterations in key regulatory hormones, namely IGF-1, T_3, insulin and leptin.

9.1.4. Bone metabolic response to short-term low EA achieved by diet or exercise independently in physically active, eumenorrheic women

The effects of low EA through diet and exercise alone on BTM have not been previously considered. In order to fill these literature gaps, in Study 3 (reported in Chapter 7) we determined and compared the effects of reduced EA at 15 kcal·kg $LBM^{-1}\cdot d^{-1}$ induced by diet or exercise independently on bone metabolism in physically active, eumenorrheic women (Figure 9.1.). The main findings of this study were that dietary-induced low EA resulted in a reduction in BT ratio, despite no differences in P1NP and β-CTX independently. Grinspoon et al. (1995) showed decreases in bone formation (OC, P1CP) and increases in bone resorption (PYD, DPD) following a 4-day fasting protocol (Grinspoon et al., 1995) (Figure 9.1.). The sedentary study population, the outdated BTM selection and the unrealistic scenario of a 4-day fasting period limit the usefulness of this study in physically active individuals. An identical energy deficit induced by exercise did not impact the BTM responses (Study 3 reported in Chapter 7). Importantly, there were no significant differences in BTM responses between the diet- and exercise-induced low EA at 15 kcal·kg $LBM^{-1}\cdot d^{-1}$. These findings suggest that implementing an energy deficit through diet or exercise does not affect markers of bone formation or resorption within at least the 3-day timeframe of the present study.

Decreases in IGF-1 and leptin were shown in both low EAs; whereas decrements in T_3 took place in the diet-induced low EA condition only, and decreases in insulin in the exercise-induced low EA condition only. When comparing the two restricted conditions, no differences in markers of calcium metabolism and regulatory hormones were shown, which is in agreement with previous findings (Koehler et al., 2016). Alterations in the aforementioned regulatory hormones occurred despite the absence of significant alterations in either bone formation or resorption; therefore, our findings do not support that these hormones mediate BTM responses, at least within the short timeframe of our study.

Conversely, the absence of changes in 17β-oestradiol levels in either the diet- or exercise induced low EA are in line with no changes in markers of bone formation and resorption in this investigation.

9.1.5. Bone metabolic response to short-term low EA achieved by diet or exercise independently in physically active combined OCP users

Given the widespread OCP use among physically active women (Bennell et al., 1999a; Burrows & Peters, 2007) and the potential for negative bone outcomes arising from their use (Liu & Lebrun, 2005), in Study 4 (reported in Chapter 8), we determined the effects of low EA achieved by diet or exercise on BTM in physically active combined OCP users (Figure 9.1). Combined OCPs were chosen over other types of hormonal contraception to allow the investigation of the effects of low EA in women using the most popular contraceptive (Lader et al., 2009). Due to the exogenously administered oestrogens, combined OCP users have down-regulated endogenous oestradiol levels (Burrows & Peters, 2007) that resemble those of postmenopausal women who are at high risk for osteoporosis (Khosla & Pacifici, 2013; Manolagas et al., 2013). Their unique oestradiol profile allows comparison with that of age-matched eumenorrheic women who experience cyclical changes in endogenous oestradiol (Elliott-Sale et al., 2013).

There were no effects of combined OCP use on BTMs in response to either diet- or exercise-induced low EA, although reduced leptin, T_3, IGF-1 levels, characteristics of compensatory responses to energy deprivation (Chapter 4; De Souza et al., 2008; Ihle & Loucks 2004), were shown. These findings suggest that changes in regulatory hormones are not related to BTM responses, at least within the 3-day experimental period. We combined the data from Study 3 (eumenorrheic women-reported in Chapter 7) and Study 4 (combined OCP users-reported in Chapter 8) to directly compare the BTM responses to low EAs between these two groups and showed no differences in any BTM marker. This absence of changes occurred despite significant differences in oestradiol levels at baseline and follow-up (combined OCP users < eumenorrheic women) and distinct alterations in oestradiol patterns (reduction in combined OCP users, no alteration in eumenorreic women) between the groups throughout the 3-day experimental period. When we directly compared the responses of regulatory hormones and markers of calcium metabolism to low EA in EU women and combined OCP users, there were no significant differences for any of these variables between the groups. Collectively, these findings do not support unfavourable effects of combined OCP use on BTMs following low EA within a 3-day experimental protocol.

Studies 3 and 4 have a number of strengths, which include the fact that all participants consumed diets of the same composition in all experimental condition, limiting the variability in macronutrient distribution between conditions and participants. All food items were weighed by investigators and were provided to the participants in order to maximise their compliance to the experimental protocol. The studies were also strengthened by the use of a crossover counterbalanced experimental design, in which each of our participants served as her own control and completed all three experimental conditions. In previous experiments, each participant has completed two (out of 4) experimental conditions to investigate the effects of distinct levels of EA (Loucks & Thuma, 2003; Ihle & Loucks, 2004).

Eumenorrheic women and combined OCP users were similar in terms of key demographic characteristics including age, body composition and fitness status, which limits confounding factors that may affect outcome variables. We purposely chose to test the eumenorrheic women during the early follicular phase of their menstrual cycle to limit the variability in BTMs due to naturally occurring cyclic changes in oestrogen and progesterone levels (Gass et al., 2008). Similarly, we also carefully selected the phase of combined OCP and matched it with the most relevant phase of the menstrual cycle in eumenorrheic women (Elliott-Sale et al., 2013). Previous research has highlighted that combined OCP use results in concentrations of endogenous oestradiol and progesterone that resemble those produced during the early follicular phase of menstrual cycle (Day 1-7) (Elliott-Sale et al., 2013; Hansen et al., 2009).

9.2. Programme impact and conclusions

This programme of work makes important contributions to the knowledge base on the impact of low EA on bone metabolism and health in physically active individuals. This has significant impact for the general public, athletes and military recruits.

An interesting finding in the present studies is that the baseline BTM levels, especially for P1NP, in our physically active, eumeorrheic women and men were towards the higher end of the reference range (in line with other data from our laboratory in similar populations). These findings suggest that the physically active individuals in our study (reported in Chapter 4) had higher bone formation than non-active populations (Jenkins et al., 2013) possibly due to osteogenic effects associated with their habitual sports involvement. In contrast, lower baseline levels of P1NP were shown in combined OCP users compared to those in eumenorrheic women, although they were within the reference range (Jenkins et al.,

2013). These findings are in line with existing literature (de Papp et al., 2007) and suggest that combined OCP users may not benefit from exercise as much as their eumeorrheic counterparts. It is likely that these discrepancies in BTM baseline levels are related with the downregulated oestradiol profile of combined OCP users, since these groups did not differ in terms of key parameters (*e.g.*, demographics, body composition, training status and markers of energy status). The robustness of these findings is reinforced by our blood sampling conditions-our participants avoided exercise the previous day and were fasted overnight prior to the early morning baseline blood sample collection. As such, we provide true baseline BTM values in physically active women (eumenorrheic and combined OCP users) and men.

Recent studies have reported that physically active females may be at great risk of low EA (Slater et al., 2016; Torstveit & Sundgot-Borgen, 2005); however, the effects of the initiation of such practices on their bone metabolism have not been explored. Study 1 (reported in Chapter 4) suggests that suboptimal dietary and exercise practices leading to low EA at 15 kcal·kg $LBM^{-1}\cdot d^{-1}$ followed even for a short period of time may reduce bone formation and increase bone resorption, thus, compromise bone health. These findings advance and add novel evidence in the Female Athlete Triad (Nattiv et al., 2007; De Souza et al., 2014) and the RED-S models (Mountjoy et al., 2014; 2015), reinforcing the need for optimal nutrition and exercise practices during a critical age for bone maintenance.

The complex interplay between energy deficiency, menstrual function and bone health in women (as the Female Athlete Triad) has been relatively well researched (Otis et al., 1997; Nattiv et al., 2007; De Souza et al., 2014) when compared with the amount of information available in physically active men. As such, the consequences of low EA on bone health in men remain poorly understood despite the recent introduction of the RED-S model (Mountjoy et al., 2014; 2015) and other reports (Tenforde et al., 2016). The current study (reported in Chapter 5) is, therefore, an important addition to the current knowledge base and showed that low EA, at 15 kcal·kg$LBM^{-1}\cdot d^{-1}$ over 5 days, had no significant effects on bone metabolism in men.

The combination of these studies allowed a direct sex comparison of BTM responses to low EA, which revealed no significant differences between physically active women and men. Although this analysis suggests that relatively to women, men may experience similar challenges regarding their bone health, further research is needed to confirm these findings. The field of the Female Athlete Triad and RED-S will greatly benefit from this analysis, which sets the basis for integrating a direct sex comparison in future research.

Conversely, the combination of Studies 1 and 2 (reported in Chapters 4 and 5) also allowed for an integrated approach of a mixed population of men and women. This has an impact for the development of educational information around feeding and training practices in arduous occupational roles to reduce the risk of skeletal injury and adverse skeletal health consequences. Some relevance might also exist concerning the physical employment standards in these occupations (Petersen et al., 2016; Blacker et al., 2015). These standards reflect the physical and physiological demands of job-related tasks, but also the risk of injury while training. There is a consensus that physical employment standards should be non-discriminatory on the grounds of sex in order to comply with existing legislation (*i.e.*, Equality Act 2010) (Petersen et al., 2016; Blacker et al., 2015). Thus, it is important to avoid bias by conducting relevant research in a mixed population of men and women, representative of the population to whom physical employment standards will apply (Milligan et al., 2016; Petersen et al., 2016).

Studies 3 and 4 (reported in Chapters 7 and 8) focused on physically active women who are more consistently affected (Chapter 4; Female Athlete Triad) by low EA in order to advance current understanding about the impact of low EA attained by exercise and diet independently. Athletic populations and military recruits often follow training routines of 1-2 hours making the exercise condition relevant to their habitual exercise practices. The diet-restricted condition, although less common among physically active populations, may occur in non-training periods (Viner et al., 2015). This condition is also representative of the intake of patients with eating disorders (*e.g.*, anorexia nervosa) or the amount of energy digested during weight loss programmes by overweight/obese individuals (Saris, 2001; Hession et al., 2009). Surprisingly, neither diet- nor exercise-induced low EA implemented for 3 days altered markers of bone formation or resorption in physically active eumoenorrheic women or combined OCP users. Our results suggest that the effects of low EA on BTMs did not differ by the way that low EA was attained, although further work is required to confirm these findings in the long-term.

The current research advances our understanding of the bone metabolic effects of low EA in combined OCP users, an overlooked sub-population of the Female Athlete Triad and the RED-S models. In addition to providing contraception, the combined OCP may offer a number of advantages for physically active women, including the relief of symptoms associated with the menstrual cycle (*e.g.*, heavy menstrual bleeding and anaemia, dysmenorrhea, menstrual migraines or acne- American College of Obstetricians and Gynaecologists, 2010; Sadler et al., 2010; Maguire & Westhoff, 2011) and flexibility with menstrual cycle around critical competitions, training or travel (Bennell et al., 1999a). These

potential benefits should be weighed against potential negative health effects, such as adverse effects of bone metabolism and health. Our results suggest that combined OCP use does not impact BTM following short-term, reduced EA and with respect to eumenorheic women, combined OCP users exhibit similar BTM responses when exposed to the same dietary and exercise conditions. As such, the current work has an impact on this debate, suggesting that combined OCP use does not compromise bone health under conditions of low EA within the short timeframe of the current study. Longer periods of low EA and recovery from exposure to low EA need to be futher investigated.

To conclude, the current programme explored the short-term effects of low EA on bone metabolism, regulatory and reproductive hormones and markers of calcium metabolism in physically active populations. This is important since individuals, such as athletes, regular exercisers or those involved in arduous occupations, often complete multiple periods of low EA induced through diet, exercise or a combination of the two, which could have cumulative effects on bone metabolism and health. The findings of the current programme of work have implications for public health programmes that aim to raise awareness of low EA and its consequences. Further implications include the provision of information relating to the early identification of those at risk of adverse skeletal consequences resulting from low EA and guidance on avoiding or managing this practice. As such, this research will be of particular interest to health care professionals and nutritionists and exercise physiologists to inform their practice.

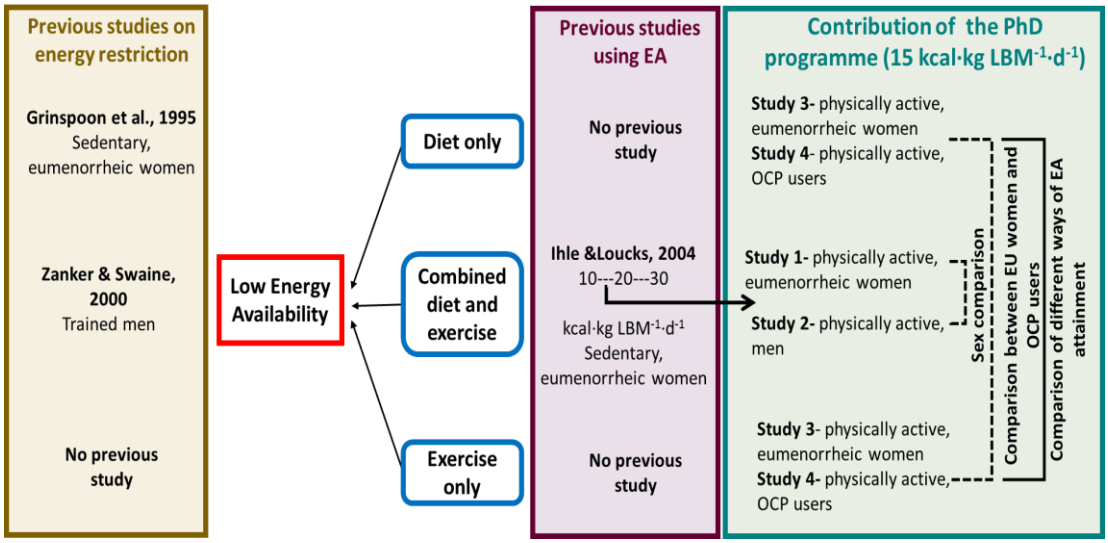


Figure 9.1. The contribution of the current PhD programme to the existing knowledge base of experimental studies in the area of EA and bone metabolism. Low EA (red box) may result from diet only, exercise only or combination of the two (blue boxes). Short-term energy restriction studies that have not been designed based on the concept of EA are summarised in the yellow box. Short-term studies that have used the concept of EA are summarised in the purple box. The green box highlights the contribution of the current PhD programme in the area of EA-bone metabolism and health in normal-weight physically active individuals. The black arrow indicates the level of low EA selected for the current programme, 15 kcal·kg $LBM^{-1}·d^{-1}$ based on previous research (Ihle & Loucks, 2004). The black elbow connector (solid line) indicates the comparison of the different ways that low EA can be attained. The black elbow connectors (dash line) indicate the combination of data to provide a sex comparison and a comparison between eumenorreic women and combined OCP users.

9.3. Limitations

There are several ways to prescribe the dietary component of low EA, with each of these ways accompanied by advantages and limitations. For example, Ihle and Loucks (2004) utilised clinical dietary products in liquid form, which provide the advantage of uniform dietary prescription, but may alter regulatory hormones (compared to solid habitual food) (Teff et al., 2010) that may in turn, affect bone metabolism. As such, in Studies 1 and 2 (reported in Chapters 4 and 6) we chose to feed participants with diets identical in macronutrient dietary composition and with food/drink items being the same as their habitual DEI in order to limit possible initial adaptations to macronutrient intake and mimic their daily lives. This also means that dietary composition was the same for CON and RES trial within participant, but was different between participants. In Studies 3 and 4, we chose

to feed our participants diets with the same food items and dietary composition allowing us to eliminate the variability in macronutrient distribution within and between participants. These diets, however, deviated to some extent from the habitual dietary composition of each participant. We decided upon this design after careful consideration of a number of factors including participants' compliance with a very low calorie diet (VLCD) (15 kcal·kgLBM·d^{-1} or ~500-600 kcal· d^{-1}), practicalities associated with (participants) preparing a VLCD and difficulties in modifying a VLCD based on habitual DEI of each participant. Designing a diet that could serve the purpose of 15 and 45 kcal· $kgLBM^{-1}·d^{-1}$ was challenging, but we were successful in including universally accepted food items that were not perceived either as too little in the diet-induced restricted condition at 15 kcal·kgLBM·d^{-1} or as too much in the controlled condition at 45 kcal· $kgLBM^{-1}·d^{-1}$.

In Studies 7 and 8, in E-RES we chose to administer the total duration of exercise in two bouts of running per day in order to allow testing to take place under supervised, laboratory conditions and enable our participants to complete the strenuous exercise throughout the 3-day experimental period. Performing two bouts of exercise on the same day may result in more pronounced metabolic responses including elevated levels of cytokines, cortisol and leucocyte count compared to the responses induced in a single exercise bout (Ronsen et al., 2001; 2002). However, repeated running sessions of similar intensity and duration separated by different recovery periods, had no differential effect on bone resorption and formation, OPG, PTH, ACa, or PO_4 in a previous experimental study (Scott et al., 2013). As such, it is unlikely that our results were affected by the way exercise was administered in terms of recovery.

In Study 8, participants were included if they were on a low-dose combined OCP, but the brand or the types of oestradiol and progestins were not specified in order to *i)* be able to recruit participants for this study and *ii)* increase the ecological validity of our results by including a representative sample of combined OCP users. Combined OCP contain fixed dosages of oestradiol and progestin, which downregulate the endogenous production of the steroids (Burrows & Peters, 2007). All participants were taking combined OCPs with different types of progestins but with the same dosage of ethinyl-oestradiol minimising the variation in this combined OCP component. Despite this, we showed substantial inter-individual variability in circulating 17β- oestradiol (Liu and Lebrun, 2005; Bennell et al., 1999a). These unexpected findings highlight the complex interactions between combined OCP and endogenous hormones, which may have contributed to the variability in BTM responses in this group. This is important finding should be considered in follow up studies.

For the purpose of similar interventions requiring rigid control of possible confounders, participants on the same combined OCPs should be recruited.

Finally, throughout the whole programme, we measured a large number of traditional (*i.e.*, insulin, leptin, T_3, IGF-1) and emerging (*i.e.*, sclerostin, GLP-2) regulatory hormones to provide insight into factors that may mediate some BTM responses. Due to high cost and time constrains, we were unable to measure others such as cortisol, PYY and ghrelin, which we would consider in future studies, as they may mediate some bone metabolic effects in response to energy deprivation (Sheid et al., 2009; Shapses & Sukumar, 2012; Ihle & Loucks, 2004) (please also see section 2.11). For the same reasons, we did not measure concentrations of reproductive hormones in our study in men, and are, therefore, unable to conclude whether or not alterations of these factors have contributed to the BTM outcomes. In women, we determined 17β-oestradiol, which is the main circulating steroid; however, testosterone may also play a role in bone metabolism and health (Manoloagas et al., 2013; Vanderschueren et al., 2014).

9.4. Future Work

This thesis expands the knowledge base on the effects of low EA on bone metabolism and health by characterising BTM responses to low EA in physically active women (eumenorrheic women and combined OCP users) and men depending on how EA is attained (diet, exercise or combination of the two) (Figure 9.1.) and provides a dataset, from which a fresh set of hypotheses can be generated and studies designed.

All studies of this PhD programme focused on the short-term ($\leq$ 5days) effects of low EA on BTM, regulatory and reproductive hormones and markers of calcium metabolism. Follow-up studies should provide further information about the effects of low EA on BTMs over a longer period. These follow-up studies would allow the assessment of additional bone-related outcomes such as BMD and bone microarchitecture. Cross-sectional studies have demonstrated distorted bone microarchitecture in amenorrheic athletes who are oestrogen, and presumably energy, deficient (Ackerman et al., 2011; 2012a). These studies cannot, however, provide a cause-effect relationship between low EA and altered bone metabolism in physically active populations. Interventions designed to detect alterations in BMD and bone microarchitecture should be longer than 12 months in duration in order to include 3 to 4 bone remodelling cycles (Heaney et al., 2000). Given these time requirements and experimental rigour, such experimental studies will be very challenging to perform due to their high cost and difficulties with participants' recruitment, compliance, and retention.

In Study 2 (reported in Chapter 5) no significant alterations in either bone formation or resorption were shown in physically active men. Individual analysis, however, revealed that some men responded with an increase in markers of bone resorption and/or a decrease in markers of bone formation. Future studies should explore if the same level of low EA implemented over a longer duration or a more severe level of reduced EA would elicit a more consistent BTM response in this population.

In Study 1, the effects of low EA (15 kcal·kgLBM^{-1}·d^{-1}) achieved through a combination of diet and exercise in physically active, eumenorrheic women were investigated. Furthermore, in Study 3 and 4 (reported in Chapter 7 and 8) we compared low EA through diet or exercise independently, but due to time and resource constraints, did not include a combined condition of diet and exercise. Although the direction and magnitude of BTM changes was similar irrespective of the way low EA was implemented, a future experiment to directly compare the effects of low EA achieved by dietary restriction, exercise, or combined dietary restriction and exercise will provide further evidence into potential differences in BTM responses to low EA implemented by different ways.

In Study 4 (reported in Chapter 8), a novel insight into the effects of low EA on BTM in combined OCP users was provided. No negative effects of low EA on markers of either bone formation or resorption were revealed in this population, despite alterations in regulatory hormones indicative of energy conservation. We cannot rule out that different doses or routes of administration of hormones might have different effects on bone turnover, which warrants further exploration. The average length of combined OCP use in our study was 5.8 years, meaning that participants were long-term users. We cannot confirm that individuals using the combined OCP for shorter or longer durations would have the same bone metabolic responses, suggesting the need for continued research.

In all studies, conventional statistics were supplemented with individual analysis. Notably, we showed considerable inter-individual variability in BTM responses to low EA. Previous research has highlighted inter-individual variability to energy deficiency in body composition changes (King et al., 2008), insulin sensitivity (King et al., 2010), and female reproductive function (Williams et al., 2015), which may be relevant to BTM responses. Further exploring and understanding inter-individual differences in bone turnover responses to low EA could provide valuable insights into the impact of EA in bone health and disease.

Although this programme focused on the effects of low EA on bone metabolism in physically active populations, the RED-S models recently acknowledged that low EA may

negatively affect a number of aspects of health and performance (Mountjoy et al., 2014; 2015). Considering the complexity of these negative consequences associated with low EA, Studies 3 and 4 (detailed in Chapter 7 and 8) were conducted on a collaborative basis with another PhD student to include more health and performance related outcomes, namely, muscle function and cognitive function (please see section 7.2.6.). The combination of these outcomes will give valuable insight into how low EA affect musculoskeletal health and performance (*i.e.*, risk for musculoskeletal injury, muscle strength). Future research to characterise the challenges associated with low EA and its interrelated effects on different systems will benefit the understanding of the Female Athlete Triad and the RED-S models and will enable the development of effective prevention and treatment strategies.

Chapter 10. References

1. Ackerman, K.E., Nazem, T., Chapko, D., Russell, M., Mendes, N., Taylor, A.P., Bouxsein, M.L., and Misra, M., 2011. Bone microarchitecture is impaired in adolescent amenorrheic athletes compared with eumenorrheic athletes and nonathletic controls. *The Journal of Clinical Endocrinology and Metabolism*, 96 (10), 3123-3133.
2. Ackerman, K.E., Patel, K.T., Guereca, G., Pierce, L., Herzog, D.B. and Misra, M., 2013b. Cortisol secretory parameters in young exercisers in relation to LH secretion and bone parameters. *Clinical endocrinology*, 78 (1), 114-119.
3. Ackerman, K.E., Pierce, L., Guereca, G., Slattery, M., Lee, H., Goldstein, M. and Misra, M., 2013a. Hip structural analysis in adolescent and young adult oligoamenorrheic and eumenorrheic athletes and nonathletes. *The Journal of Clinical Endocrinology and Metabolism*, 98 (4), 1742-1749.
4. Ackerman, K.E., Putman, M., Guereca, G., Taylor, A.P, Pierce, L., Herzog, D.B., Klibanski, A., Bouxsein, M.L., and Misra, M., 2012a. Cortical microstructure and estimated bone strength in young amenorrheic athletes, eumenorrheic athletes and non-athletes. *Bone*, 51 (4), 680-687.
5. Ackerman, K.E., Skrinar. G.S., Medvedova, E., Misra, M., and Miller, K.K., 2012b. Estradiol levels predict bone mineral density in male collegiate athletes: a pilot study. *Clinical Endocrinology (Oxf)*, 76 (3), 339-345.
6. ACOG Practice Bulletin No. 110: noncontraceptive uses of hormonal contraceptives, *Obstetrics & Gynecology*, 2010, 115 (1), 206–218.
7. Aerenhouts, D., Clarys, P., Taeymans, J. and Van Cauwenberg, J., 2015. Estimating Body Composition in Adolescent Sprint Athletes: Comparison of Different Methods in a 3 Years Longitudinal Design. *PloS one*, 10 (8), e0136788.
8. Ahlborg, H.G., Johnell, O., Turner, C.H., Rannevik, G., and Karlsson, M.K., 2003. Bone loss and bone size after menopause. *New England Journal of Medicine*, 349 (4), 327-334.
9. Aikawa, Y., Agata, U., Kakutani, Y., Higano, M., Hattori, S., Ogata, H., Ezawa, I. and Omi, N., 2015. The interaction of voluntary running exercise and food restriction induces low bone strength and low bone mineral density in young female rats. *Calcified tissue international*, 97 (1), 90-99.
10. Åkesson, K., Marsh, D., Mitchell, P.J., McLellan, A.R., Stenmark, J., Pierroz, D.D., Kyer, C., Cooper, C. and IOF Fracture Working Group, 2013. Capture the Fracture: a Best Practice Framework and global campaign to break the fragility fracture cycle. *Osteoporosis International*, 24 (8), 2135-2152.
11. Alajmi, N., Deighton, K., King, J.A., Reischak-Oliveira, A., Wasse, L.K., Jones, J., Batterham, R.L., and Stensel, D.J., 2016. Appetite and Energy Intake Responses to Acute Energy Deficits in Females versus Males. *Medicine and Science in Sports and Exercise*, 48 (3), 412-420.
12. Armamento-Villareal, R., Sadler, C., Napoli, N., Shah, K., Chode, S., Sinacore, D.R., Qualls, C., and Villareal, D.T., 2012. Weight loss in obese older adults increases serum sclerostin and impairs hip geometry but both are prevented by exercise training. *Journal of Bone and Mineral Research*, 27 (5), 1215-1221.

13. Armstrong, V.J., Muzylak, M., Sunters, A., Zaman, G., Saxon, L.K., Price, J.S. and Lanyon, L.E., 2007. Wnt/β-catenin signaling is a component of osteoblastic bone cell early responses to load-bearing and requires estrogen receptor α. *Journal of Biological Chemistry*, 282(28), 20715-20727.
14. Atkinson, G. and Nevill, A.M., 1998. Statistical methods for assessing measurement error (reliability) in variables relevant to sports medicine. *Sports medicine*, 26 (4), 217-238.
15. Bailey, D.A., McKay, H.A., Mirwald, R.L., Crocker, P.R. and Faulkner R.A., 1999. A six-year longitudinal study of the relationship of physical activity to bone mineral accrual in growing children: University of Saskatchewan Bone Mineral Accrual Study. *Journal of Bone and Mineral Research*, 14 (10), 1672–1679.
16. Baim, S., Binkley, N., Bilezikian, J.P., Kendler, D.L., Hans, D.B., Lewiecki, E.M., and Silverman, S., 2008. Official Positions of the International Society for Clinical Densitometry and executive summary of the 2007 ISCD Position Development Conference. *Journal of Clinical Densitometry*, 11 (1), 75-91.
17. Barrack, M.T., Gibbs, J.C., De Souza, M.J., Williams, N.I., Nichols, J.F., Rauh, M.J., and Nattiv, A., 2014. Higher incidence of bone stress injuries with increasing female athlete triad-related risk factors: a prospective multisite study of exercising girls and women. *American Journal of Sports Medicine*, 42 (4), 949-958.
18. Barrack, M.T., Rauh, M.J., Barkai, H.S. and Nichols, J.F., 2008. Dietary restraint and low bone mass in female adolescent endurance runners. *American Journal of clinical nutrition*, 87(1), 36-43.
19. Barrack, M.T., Van Loan, M.D., Rauh, M.J. and Nichols, J.F., 2010. Physiologic and behavioral indicators of energy deficiency in female adolescent runners with elevated bone turnover. *American Journal of Clinical Nutrition*, 92 (3), 652-659.
20. Bartell, S.M., Han, L., Kim, H.N., Kim, S.H., Katzenellenbogen, J.A., Katzenellenbogen, B.S., Chambliss, K.L., Shaul, P.W., Roberson, P.K., Weinstein, R.S., Jilka, R.L., Almeida, M. and Manolagas S.C., 2013. Non-nuclear-initiated actions of the estrogen receptor protect cortical bone mass. *Molecular Endocrinology*, 27 (4), 649-656.
21. Bauer, D., Krege, J., Lane, N., Leary, E., Libanati, C., Miller, P., Myers, G., Silverman, S., Vesper, H.W., Lee, D., Payette, M. and Randall, S., 2012. National Bone Health Alliance Bone Turnover Marker Project: current practices and the need for US harmonization, standardization, and common reference ranges. *Osteoporosis International*, 23 (10), 2425-2433.
22. Baxter-Jones A.D., Faulkner R.A., Forwood, M.R. Mirwald, R.L. Bailey D.A., 2011. Bone mineral accrual from 8 to 30 years of age: an estimation of peak bone mass. *Journal of Bone and Mineral Research*, 26 (8), 1729–1739.
23. Beato, M., and Klug, J., 2000. Steroid hormone receptors: an update. *Human Reproduction Update*, 6 (3), 225–236.
24. Beck, T.J., 2007. Extending DXA beyond bone mineral density: understanding hip structure analysis. *Current Osteoporosis Reports*, 5 (2), 49-55.

25. Beck, T.J., Ruff, C.B., Shaffer, R.A., Betsinger, K., Trone, D.W. and Brodine, S.K., 2000. Stress fracture in military recruits: gender differences in muscle and bone susceptibility factors. *Bone*, 27 (3), 437-444.
26. Belavý, D.L., Baecker, N., Armbrecht, G., Beller, G., Buehlmeier, J., Frings-Meuthen, P., Rittweger, J., Roth, H.J., Heer, M. and Felsenberg, D., 2016. Serum sclerostin and DKK1 in relation to exercise against bone loss in experimental bed rest. *Journal of Bone and Mineral Metabolism*, 34 (3), 354-365.
27. Bellows, M., Tanguay, J., Crouse, S.F. and Harbourt, D., 2013. Vitamin D Deficiency in TAMU Female Basketball Players and Supplement Effectiveness. In International Journal of Exercise Science: Conference Proceedings, 2 (5), 19.
28. Bennell, K., Matheson, G., Meeuwisse, W. and Brukner, P., 1999b. Risk factors for stress fractures. *Sports Medicine*, 28 (2), 91-122.
29. Bennell, K., White, S. and Crossley K., 1999a. The oral contraceptive pill: a revolution for sportswomen? *British Journal of Sports Medicine*, 33 (4), 231-238.
30. Bennell, K.L., Brukner, P.D. and Malcolm, S.A., 1996b. Effect of altered reproductive function and lowered testosterone levels on bone density in male endurance athletes. *British Journal of Sports Medicine*, 30 (3), 205–208.
31. Bennell, K.L., Malcolm, S.A., Thomas, S.A., Reid, S.J., Brukner, P.D., Ebeling, P.R. and Wark, J.D., 1996a. Risk factors for stress fractures in track and field athletes A twelve-month prospective study. *American Journal of Sports Medicine*, 24 (6), 810-818.
32. Berger, C., Goltzman, D., Langsetmo, L., Joseph, L., Jackson, S., Kreiger, N., Tenenhouse, A., Davison, K.S., Josse, R.G., Prior, J.C., Hanley, D.A. and CaMos Research Group, 2010. Peak bone mass from longitudinal data: implications for the prevalence, pathophysiology, and diagnosis of osteoporosis. *Journal of Bone and Mineral Research*, 25 (9), 1948–1957.
33. Bergström, I., Crisby, M., Engström, A.M., Hölcke, M., Fored, M., Jakobsson Kruse, P. and Sandberg, A.M., 2013. Women with anorexia nervosa should not be treated with estrogen or birth control pills in a bone- sparing effect. Acta obstetricia et gynecologica Scandinavica, 92 (8), 877-880.
34. Bianco, P., Robey, P.G., Saggio, I. and Riminucci, M., 2010. Mesenchymal" stem cells in human bone marrow (skeletal stem cells): a critical discussion of their nature, identity, and significance in incurable skeletal disease. *Human Gene Therapy*, 21 (9), 1057-1066.
35. Bilezikian, J.P., Morishima, A., Bell, J. and Grumbach, M.M., 1998. Increased bone mass as a result of estrogen therapy in a man with aromatase deficiency. *New England Journal of Medicine*, 339 (9), 599-603.
36. Bischoff-Ferrari, H.A., Dawson-Hughes, B., Staehelin, H.B., Orav, J.E., Stuck, A.E., Theiler, R., Wong, J.B., Egli, A., Kiel, D.P. and Henschkowski, J., 2009. Fall prevention with supplemental and active forms of vitamin D: a meta-analysis of randomised controlled trials. *British Medical Journal*, 1, 339:b3692.

37. Bjarnason, N.H., Henriksen, E.E., Alexandersen, P., Christgau, S., Henriksen, D.B. and Christiansen, C., 2002. Mechanism of circadian variation in bone resorption. *Bone*, 30 (1), 307–313.
38. Black, A.J., Topping, J., Durham, B., Farquharson, R.G. and Fraser, W.D., 2000. A detailed assessment of alterations in bone turnover, calcium homeostasis, and bone density in normal pregnancy. *Journal of Bone and Mineral Research*, 15 (3), 557-563.
39. Blacker, S.D., Rayson, M.P., Wilkinson, D.M., Carter, J.M., Nevill, A.M. and Richmond, V.L., 2015. Physical employment standards for UK fire and rescue service personnel. *Occupational medicine*, p.kqv122.
40. Blackmore, K.M., Wong, J. and Knight J.A., 2011. A cross-sectional study of different patterns of oral contraceptive use among premenopausal women and circulating IGF-1: implications for disease risk. *BMC Women's Health*, 11 (1), 15.
41. Bland, J.M. and Altman, D.G.,1986. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet, 1 (8476), 307-310.
42. Bonjour, J.P., 2005. Dietary protein: an essential nutrient for bone health. *Journal of the American College of Nutrition*, 24 (sup6), 526S-536S.
43. Bonjour, J.P., Carrie, A.L., Ferrari, S., Clavien, H., Slosman, D., Theintz, G. and Rizzoli. R., 1997. Calcium-enriched foods and bone mass growth in prepubertal girls: a randomized, double-blind, placebo-controlled trial. *Journal of Clinical Investigation*, 99 (6), 1287-1294.
44. Bonnet, N. and Ferrari, S.L., 2010. Exercise and the skeleton: How it works and what it really does. *International Bone and Mineral Society BoneKEy*, 7 (7), 235–248.
45. Boreham, C.A. and McKay, H.A., 2011. Physical activity in childhood and bone health. *British Journal of Sports Medicine*, 45 (11), 877-879.
46. Borer, K.T., 2005. Physical activity in the prevention and amelioration of osteoporosis in women: interaction of mechanical, hormonal and dietary factors. *Sports Medicine*, 35(9), 779-830.
47. Bouxsein, M.L., 2005. Determinants of skeletal fragility. *Best Practice Research and Clinical Rheumatology*, 19 (6), 897-911.
48. Brahm, H., Strom, H., Piehl-Aulin, K., Mallmin, H. and Ljunghall, S., 1997. Bone metabolism in endurance trained athletes: a comparison to population-based controls based on DXA, SXA, quantitative ultrasound, and biochemical markers. *Calcified Tissue International*, 61 (6), 448–454.
49. Brown, J.P., Albert, C., Nassar, B.A., Adachi, J.D., Cole, D., Davison, K.S., Dooley, K.C., Don-Wauchope, A., Douville, P., Hanley, D.A., Jamal, S.A., Josse, R, Kaiser, S., Krahn, J., Krause, R., Kremer, R., Lepage, R., Letendre, E., Morin, S., Ooi, D.S., Papaioaonnou, A., Ste-Marie, L.G., 2009. Bone turnover markers in the management of postmenopausal osteoporosis. *Clinical Biochemistry*, 42 (10-11), 929-942.
50. Burghardt, A.J., Kazakia, G.J., Sode, M., de Papp, A.E., Link, T.M. and Majumdar, S., 2010. A longitudinal HR-pQCT study of alendronate treatment in postmenopausal women with low bone density: Relations among density, cortical and trabecular microarchitecture, biomechanics, and bone turnover. *Journal of Bone and Mineral Research*, 25 (12), 2558-2571.

51. Burkman, R., Bell, C. and Serfaty, D., 2011. The evolution of combined oral contraception: improving the risk-to-benefit ratio. *Contraception*, 84 (1), 19–34.
52. Burr, D.B., 2002. Targeted and non-targeted remodelling. *Bone* 30 (1), 2-4.
53. Burr, D.B., Yoshikawa, T., Teegarden, D., Lyle, R., McCabe, G., McCabe, L.D. and Weaver, C.M., 2000. Exercise and oral contraceptive use suppress the normal age-related increase in bone mass and strength of the femoral neck in women 18–31 years of age. *Bone*, 27 (6), 855-863.
54. Burrows, M. and Peters, C.E., 2007. The influence of oral contraceptives on athletic performance in female athletes. *Sports medicine*, 37 (7), 557-574.
55. Canalis, E.R, Mazziotti, G., Giustina, A. and Bilezikian, J.P., 2007. Glucocorticoid-induced osteoporosis: pathophysiology and therapy. *Osteoporosis International*, 18 (10), 1319-1328.
56. Canalis, E.R., Rydziel, S.H., Delany, A.M., Varghese, S.A., Jeffrey, J.J., 1995. Insulin-like growth factors inhibit interstitial collagenase synthesis in bone cell cultures. *Endocrinology*, 136 (4), 1348-54.
57. Cann, C.E., Martin, M.C., Genant, H.K. and Jaffe, R.B., 1984. Decreased spinal mineral content in amenorrheic women. *The Journal of the American Medical Association*, 251 (5), 626-629.
58. Cano Sokoloff, N., Eguiguren, M.L., Wargo, K., Ackerman, K.E., Baskaran, C., Singhal, V., Clarke, H., Slattery, M., Lee, H., Eddy, K.T. and Misra, M, 2015. Bone parameters in relation to attitudes and feelings associated with disordered eating in oligo-amenorrheic athletes, eumenorrheic athletes, and nonathletes. *International Journal of Eating Disorders*, 48 (5), 522-526.
59. Caporaso, F., Frisch, F. and Sumida, K.D., 2011. Compromised bone health in non-obese, older women with low caloric intake. *Journal of Community Health*, 36 (4), 559-564.
60. Carani, C., Qin, K., Simoni, M., Faustini-Fustini, M., Serpente, S., Boyd, J., Korach, K.S. and Simpson, E.R.., 1997. Effect of testosterone and estradiol in a man with aromatase deficiency. *New England Journal of Medicine*, 337 (2), 91-95.
61. Carnevale, V., Romagnoli, E., Cipriani, C., Del Fiacco, R., Piemonte, S., Pepe, J., Scillitani, A. and Minisola, S., 2010. Sex hormones and bone health in males. *Archives of Biochemistry and Biophysics*, 503 (1), 110-117.
62. Carr, B.R. (1998). Uniqueness of oral contraceptive progestins. *Contraception*, 58 (3 Suppl), 23S-27S.
63. Carter, D.R., Bouxsein, M.L. and Marcus, R., 1992. New approaches for interpreting projected bone densitometry data. *Journal of bone and mineral research*, 7(2), 137-145.
64. Cauley, J.A., Lui, L.Y., Barnes, D., Ensrud, K.E., Zmuda, J.M., Hillier, T.A., Hochberg, M.C., Schwartz, A.V., Yaffe, K., Cummings, S.R., Newman, A.B. and SOF Research Group, 2009. Successful skeletal aging: a marker of low fracture risk and longevity. The Study of Osteoporotic Fractures (SOF). *Journal of Bone and Mineral Research*, 24 (1), 134-143.
65. Center, J.R., Nguyen, T.V., Schneider, D., Sambrook, P.N. and Eisman, J.A., 1999. Mortality after all major types of osteoporotic fracture in men and women: an observational study. *The Lancet*, 353 (9156), 878-882.

66. Chan, J.L. and Mantzoros, C.S., 2005. Role of leptin in energy-deprivation states: normal human physiology and clinical implications for hypothalamic amenorrhoea and anorexia nervosa. *The Lancet*, 366 (9479), 74-85.
67. Chao, D., Espeland, M.A., Farmer, D., Register, T.C., Lenchik, L., Applegate, W.B. and Ettinger, W.H., 2000. Effect of voluntary weight loss on bone mineral density in older overweight women. *Journal of American Geriatric Society*, 48 (7), 753–759.
68. Chapurlat, R.D. and Confavreux, C.B., 2016. Novel biological markers of bone: from bone metabolism to bone physiology. *Rheumatology (Oxford)*, pii: kev410.
69. Chevalley, T., Rizzoli, R., Hans, D., Ferrari, S. and Bonjour, J.P., 2005. Interaction between calcium intake and menarcheal age on bone mass gain: an eight-year follow-up study from prepuberty to postmenarche. *Journal of Bone and Mineral Metabolism*, 90 (1), 44-51.
70. Christo, K., Prabhakaran, R., Lamparello, B., Cord, J., Miller, K.K., Goldstein, M.A., Gupta, N., Herzog, D.B., Klibanski, A. and Misra, M., 2008. Bone metabolism in adolescent athletes with amenorrhea, athletes with eumenorrhea, and control subjects. *Pediatrics*, 121 (6), 1127-1136.
71. Cibula, D., Skrenkova, J., Hill, M. and Stepan, J.J., 2012. Low-dose estrogen combined oral contraceptives may negatively influence physiological bone mineral density acquisition during adolescence. *European Journal of Endocrinology*, 166 (6), 1003-111.
72. Clarke, B.L. and Khosla, S., 2010. Female reproductive system and bone. *Archives of Biochemistry and Biophysics*, 503 (1), 118-128.
73. Cline, A.D., Jansen, G.R. and Melby, C.L., 1998. Stress fractures in female army recruits: implications of bone density, calcium intake, and exercise. *Journal of the American College of Nutrition*, 17 (2), 128-135.
74. Clowes, J. A., Hannon, R. A., Yap, T. S., Hoyle, N. R., Blumsohn, A. and Eastell, R., 2002. Effect of feeding on bone turnover markers and its impact on biological variability of measurements. *Bone*, 30 (6), 886-890.
75. Cobb, K.L., Bachrach, L.K., Greendale, G.A., Marcus, R.O., Neer, R.M., Nieves, J.E., Sowers, M.F., Brown, Jr.B.W., Gopalakrishna, G., Luetters, C. and Tanner, H.K., 2003. Disordered eating, menstrual irregularity, and bone mineral density in female runners. *Medicine and Science in Sports and Exercise*, 35 (5), 711-719.
76. Cobb, K.L., Bachrach, L.K., Sowers, M., Nieves, J., Greendale, G.A., Kent, K.K., Brown, B.W., Pettit, K., Harper, D.M. and Kelsey, J.L., 2007. The effect of oral contraceptives on bone mass and stress fractures in female runners. *Medicine and Science in Sports and Exercise*, 39 (9), 1464-1467.
77. Compston, J.E., Watts, N.B., Chapurlat, R., Cooper, C., Boonen, S., Greenspan, S., Pfeilschifter, J., Silverman, S., Díez-Pérez, A., Lindsay, R. and Saag, K.G., 2011. Obesity is not protective against fracture in postmenopausal women: GLOW. *The American Journal of Medicine*, 124 (11), 1043-1050.
78. Compston, J.E., Wyman, A., FitzGerald, G., Adachi, J.D., Chapurlat, R.D., Cooper, C., Díez-Pérez, A., Gehlbach, S.H., Greenspan, S.L., Hooven, F.H., LaCroix, A.Z., March, L., Netelenbos, J.C., Nieves, J.W., Pfeilschifter, J., Rossini, M., Roux, C., Saag, K.G., Siris, E.S., Silverman, S.,

Watts, N.B. and Anderson, F.A., 2016. Increase in Fracture Risk Following Unintentional Weight Loss in Postmenopausal Women: The Global Longitudinal Study of Osteoporosis in Women. *Journal of Bone and Mineral Research*, 31 (7), 1466-1472.

79. Conde, J., Scotece, M., Gómez, R., Gómez-Reino, J.J., Lago, F. and Gualillo, O., 2010. At the crossroad between immunity and metabolism: focus on leptin. *Expert Reviews of Clinical immunology*, 6 (5), 801-808.
80. Conigrave, A.D., Brown, E.M. and Rizzoli, R., 2008. Dietary protein and bone health: roles of amino acid-sensing receptors in the control of calcium metabolism and bone homeostasis. *Annual Reviews of Nutrition*, 28, 131-155.
81. Cornish, J., Callon, K.E. and Reid, I.R., 1996. Insulin increases histomorphometric indices of bone formation in vivo. *Calcified Tissue International*, 59 (6), 492-495.
82. Cornish, J., Callon, K.E., Bava, U., Lin, C., Naot, D., Hill, B.L., Grey, A.B., Broom, N., Myers, D.E., Nicholson, G.C. and Reid, I.R., 2002. Leptin directly regulates bone cell function in vitro and reduces bone fragility in vivo. *Journal of Endocrinology*, 175 (2), 405-415.
83. Craig, C.L., Marshall, A.L., Sjöström, M., Bauman, A.E., Booth, M.L., Ainsworth, B.E., Pratt, M., Ekelund, U., Yngve, A., Sallis, J.F. and Oja, P., 2003. International Physical Activity Questionnaire 12-country reliability and validity. *Medicine and Science in Sports and Exercise*, 35 (8), 1381-1395.
84. Crockett, J.C., Rogers, M.J., Coxon, F.P., Hocking, L.J. and Helfrich, M.H., 2011. Bone remodelling at a glance. *Journal of Cell Science*, 124 (Pt 7), 991-998.
85. Crouter, S.E., Clowers, K.G., and Bassett, D.R., 2006. A novel method for using accelerometer data to predict energy expenditure. *Journal of Applied Physiology*, 100 (4), 1324-1331.
86. Curtis, K.M., and Martins, S.L. 2006. Progestogen-only contraception and bone mineral density: a systematic review. Contraception, 73 (5), 470-487.
87. Darling, A.L., Millward, D.J., Torgerson, D.J., Hewitt, C.E. and Lanham-New, S.A., 2009. Dietary protein and bone health: a systematic review and meta-analysis. *American Journal of Clinical Nutrition*, 90 (6), 1674-1692.
88. Datir, A.P., Saini, A., Connell, D. and Saifuddin, A., 2007. Stress-related bone injuries with emphasis on MRI. *Clinical radiology*, 62 (9), 828-836.
89. de Papp, A.E., Bone, H.G., Caulfield, M.P., Kagan, R., Buinewicz, A., Chen, E., Rosenberg, E. and Reitz, R.E., 2007. A cross-sectional study of bone turnover markers in healthy premenopausal women. *Bone*, 40 (5), 1222-1230.
90. De Souza, M.J. and Williams, N.I., 2005. Beyond hypoestrogenism in amenorrheic athletes: energy deficiency as a contributing factor for bone loss. *Current Sports Medicine Reports*, 4 (1), 38-44.
91. De Souza, M.J., Arce, J.C., Pescatello, L.S., Scherzer, H.S. and Luciano, A.A., 1994. Gonadal hormones and semen quality in male runners. *International Journal of Sports Medicine*, 15 (07), 383-391.
92. De Souza, M.J., Miller, B.E., Sequenzia, L.C., Luciano, A.A., Ulreich, S., Stier, S., Prestwood, K. and Lasley, B.L., 1997. Bone Health Is Not Affected by Luteal Phase Abnormalities and

Decreased Ovarian Progesterone Production in Female Runners. *The Journal of Clinical Endocrinology and Metabolism*, 82 (9), 2867-2876.

93. De Souza, M.J., Nattiv, A., Joy, E., Misra, M., Williams, N.I., Mallinson, R.J., Gibbs, J.C., Olmsted, M., Goolsby, M., Matheson, G., Female Athlete Triad Coalition, American College of Sports Medicine, American Medical Society for Sports Medicine and American Bone Health Alliance, 2014a. 2014 Female Athlete Triad Coalition consensus statement on treatment and return to play of the female athlete triad: 1st International Conference held in San Francisco, CA, May 2012, and 2nd International Conference held in Indianapolis, IN, May 2013. *Clinical Journal of Sport Medicine*, 24 (2), 96-119.
94. De Souza, M.J., Toombs, R.J., Scheid, J.L., O'Donnell, E., West, S.L. and Williams, N.I., 2010. High prevalence of subtle and severe menstrual disturbances in exercising women: confirmation using daily hormone measures. *Human reproduction*, 25 (2), 491-503.
95. De Souza, M.J., Van Heest, J., Demers, L.M. and Lasley, B.L., 2003. Luteal phase deficiency in recreational runners: evidence for a hypometabolic state. *Clinical Journal of Sport Medicine*, 88 (1), 337-346.
96. De Souza, M.J., West, S.L., Jamal, S.A., Hawker, G.A., Gundberg, C.M. and Williams, N.I., 2008. The presence of both an energy deficiency and estrogen deficiency exacerbate alterations of bone metabolism in exercising women. *Bone*, 43 (1), 140-148.
97. De Souza, M.J., Williams, N.I., Nattiv, A., Joy, E., Misra, M., Loucks, A.B., Matheson, G., Olmsted, M.P., Barrack, M., Mallinson, R.J., Gibbs, J.C., Goolsby, M., Nichols, J.F., Drinkwater, B., Sanborn, C., Agostini, R., Otis, C.L., Johnson, M.D., Hoch, A.Z., Alleyne, J.M., Wadsworth, L.T., Koehler, K., VanHeest, J., Harvey, P., Kelly, A.K., Fredericson, M., Brooks, G.A., O'Donnell, E., Callahan, L.R., Putukian, M., Costello, L., Hecht, S., Rauh, M.J., McComb, J., 2014b. Misunderstanding the female athlete triad: refuting the IOC consensus statement on Relative Energy Deficiency in Sport (RED-S). *British Journal of Sports Medicine*, 48 (20), 1461–1465.
98. Delhanty, P.J.D., van der Eerden, B.C.J., van der Velde, M., Gauna, C., Pols, H.A.P., Jahr, H., Chiba, H., van der Lely, A.J. and van Leeuwen, J.P.T.M., 2006. Ghrelin and unacylated ghrelin stimulate human osteoblast growth via mitogen-activated protein kinase (MAPK)/phosphoinositide 3-kinase (PI3K) pathways in the absence of GHS-R1a. *Journal of Endocrinology*, 188 (1), 37-47.
99. DeLuca, H.F., 2004. Overview of general physiologic features and functions of vitamin D. *The American Journal of Clinical Nutrition*, 80 (6), 1689S-1696S.
100. Devlin, M.J., Cloutier, A.M., Thomas, N.A., Panus, D.A., Lotinun, S., Pinz, I., Baron, R., Rosen, C.J. and Bouxsein, M.L., 2010. Caloric restriction leads to high marrow adiposity and low bone mass in growing mice. *Journal of Bone and Mineral Research*, 25 (9), 2078-2088.
101. DiMarco, N.M., Dart, L. and Sanborn, C.B., 2007. Modified activity-stress paradigm in an animal model of the female athlete triad. *Journal of Applied Physiology*, 103 (5), 1469-1478.
102. Diez-Perez, A., Güerri, R., Nogues, X., Cáceres, E., Peña, M. J., Mellibovsky, L., Randall, C., Bridges, D., Weaver, J.C., Proctor, A., Brimer, D., Koester, K.J., Ritchie, R. and Hansma, P.K.

2010. Microindentation for In Vivo Measurement of Bone Tissue Mechanical Properties in Humans. *Journal of Bone and Mineral Research*, 25 (8), 1877–1885.

103. Dimitri, P., Wales, J.K. and Bishop, N., 2010. Fat and bone in children: differential effects of obesity on bone size and mass according to fracture history. *Journal of Bone and Mineral Research*, 25 (3), 527-536.

104. Dolan, E., Crabtree, N., McGoldrick, A., Ashley, D.T., McCaffrey, N. and Warrington, G.D., 2011. Weight regulation and bone mass: a comparison between professional jockeys, elite amateur boxers, and age, gender and BMI matched controls. *Journal of Bone and Mineral Metabolism*, 30 (2), 164–170.

105. Dolan, E., McGoldrick, A., Davenport, C., Kelleher, G., Byrne, B., Tormey, W., Smith, D. and Warrington, G.D., 2012. An altered hormonal profile and elevated rate of bone loss are associated with low bone mass in professional horse-racing jockeys. *Journal of Bone and Mineral and Metabolism*, 30 (5), 534–542.

106. Donato, J., Cravo, R.M., Frazão, R. and Elias, C.F., 2010. Hypothalamic sites of leptin action linking metabolism and reproduction. *Neuroendocrinology*, 93 (1), 9-18.

107. Donescu, O.S., Battié, M.C., Kaprio, J., Levalahti, E., Risteli, J., Eyre, D. and Videman, T., 2007. Genetic and constitutional influences on bone turnover markers: a study of male twin pairs. Calcified tissue international, 80 (2), 81-88.

108. Drinkwater, B. L., Nilson, K., Chesnut III, C. H., Bremner, W. J., Shainholtz, S., and Southworth, M. B., 1984. Bone mineral content of amenorrheic and eumenorrheic athletes. New England Journal of Medicine, 311 (5), 277-281.

109. Drinkwater, B.L., Bruemner, B. and Chesnut, C.H., 1990. Menstrual history as a determinant of current bone density in young athletes. Jama, 263 (4), 545-548.

110. Duan, Y., Parfitt, A.M. and Seeman, E., 1999. Vertebral bone mass, size, and volumetric density in women with spinal fractures. Journal of bone and mineral research, 14(10), 1796-1802.

111. Duckham, R.L., Peirce, N., Meyer, C., Summers, G.D., Cameron, N. and Brooke-Wavell, K., 2012. Risk factors for stress fracture in female endurance athletes: a cross-sectional study. *BMJ Open*, 2 (6), p.e001920.

112. Ducy, P., Amling, M., Takeda, S., Priemel, M., Schilling, A.F., Beil, F.T., Shen, J., Vinson, C., Rueger, J.M. and Karsenty, G., 2000. Leptin inhibits bone formation through a hypothalamic relay: A central control of bone mass. *Cell*, 100 (2), 197–207.

113. Elliott-Sale, K.J., Smith, S., Bacon, J., Clayton, D., McPhilimey, M., Goutianos, G., Hampson, J. and Sale, C., 2013. Examining the role of oral contraceptive users as an experimental and/or control group in athletic performance studies. *Contraception*, 88 (3), 408-412.

114. Engelke, K., Adams, J.E., Armbrecht, G., Augat, P., Bogado, C.E., Bouxsein, M.L., Felsenberg, D., Ito, M., Prevrhal, S., Hans, D.B. and Lewiecki, E.M., 2008. Clinical use of quantitative computed tomography and peripheral quantitative computed tomography in the management of osteoporosis in adults: the 2007 ISCD Official Positions. *Journal of Clinical Densitometry*, 11 (1), 123-162.

115. Evans, A.L., Paggiosi, M.A., Eastell, R. and Walsh, J.S., 2015. Bone density, microstructure and strength in obese and normal weight men and women in younger and older adulthood. *Journal of Bone and Mineral Research*, 30 (5), 920-928.
116. Evans, R.K., Antczak, A.J., Lester, M., Yanovich, R., Israeli, E. and Moran, D.S., 2008. Effects of a 4-month recruit training program on markers of bone metabolism. *Medicine and Science in Sports and Exercise*, 1 (Suppl), 660S-670S.
117. Faje, A.T., Karim, L., Taylor, A., Lee, H., Miller, K.K., Mendes, N., Meenaghan, E., Goldstein, M.A., Bouxsein, M.L., Misra, M. and Klibanski, A., 2013. Adolescent girls with anorexia nervosa have impaired cortical and trabecular microarchitecture and lower estimated bone strength at the distal radius. *The Journal of Clinical Endocrinology and Metabolism*, 98 (5), 1923-1929.
118. Falahati-Nini, A., Riggs, B.L., Atkinson, E.J., O'Fallon, W.M., Eastell, R. and Khosla, S., 2000. Relative contributions of testosterone and estrogen in regulating bone resorption and formation in normal elderly men. *Journal of Clinical Investigation*, 106 (12), 1553-1560.
119. Fallon, K.E., Fallon, S.K. and Boston, T., 2001. The acute phase response and exercise: court and field sports. *British Journal of Sports Medicine*, 35 (3), 170-173.
120. Fazeli, P.K. and Klibanski, A., 2014. Anorexia nervosa and bone metabolism. *Bone*, 66, 39-45.
121. Feldman, H.A., Longcope, C., Derby, C.A., Johannes, C.B., Araujo, A.B., Coviello, A.D., Bremner, W.J. and McKinlay, J.B., 2002. Age trends in the level of serum testosterone and other hormones in middle-aged men: longitudinal results from the Massachusetts male aging study. *The Journal of Clinical Endocrinology and Metabolism*, 87 (2), 589-598.
122. Felson, D.T., Zhang, Y., Hannan, M.T. and Anderson, J.J., 1993. Effects of weight and body mass index on bone mineral density in men and women: the Framingham study. *Journal of Bone and Mineral Research*, 8 (5), 567–573.
123. Feng, X. and McDonald, J.M., 2011. Disorders of bone remodeling. *Annual Reviews of Pathology*, 6, 121-145.
124. Ferrari S., 2008. Human genetics of osteoporosis. *Best Practice in Research and Clinical Endocrinology and Metabolism*, 22 (5), 723-735.
125. Finestone, A., Milgrom, C., Wolf, O., Petrov, K., Evans, R. and Moran, D., 2011. Epidemiology of metatarsal stress fractures versus tibial and femoral stress fractures during elite training. *Foot and ankle International*, 32 (1), 16-20.
126. Finkelstein, J.S., Sowers, M., Greendale, G.A., Lee, M.L.T., Neer, R.M., Cauley, J.A. and Ettinger, B., 2002. Ethnic variation in bone turnover in pre-and early perimenopausal women: effects of anthropometric and lifestyle factors. *The Journal of Clinical Endocrinology and Metabolism*, 87 (7), 3051-3056.
127. Fontana, L., Villareal, D.T., Das, S.K., Smith, S.R., Meydani, S.N., Pittas, A.G., Klein, S., Bhapkar, M., Rochon, J., Ravussin, E., Holloszy, J.O. and CALERIE Study Group, 2016. Effects of 2-year calorie restriction on circulating levels of IGF-1, IGF-binding proteins and cortisol in nonobese men and women: a randomized clinical trial. *Aging Cell*, 15 (1), 22-27.

128. Fontana, L., Weiss, E.P., Villareal, D.T., Klein, S. and Holloszy, J.O., 2008. Long-term effects of calorie or protein restriction on serum IGF-1 and IGFBP-3 concentration in humans. *Aging Cell*, 7 (5), 681-687.
129. Fredericson, M. and Kent, K.., 2005. Normalization of bone density in a previously amenorrheic runner with osteoporosis. *Medicine and Science in Sports and Exercise*, 37 (9), 1481-1486.
130. Fredericson, M., Chew, K., Ngo, J., Cleek, T., Kiratli, J. and Cobb, K., 2007. Regional bone mineral density in male athletes: a comparison of soccer players, runners and controls. *British Journal of Sports Medicine*, 41 (10), 664-668.
131. Fredericson, M., Jennings, F., Beaulieu, C. and Matheson, G.O., 2006. Stress fractures in athletes. *Topics in Magnetic Resonance Imaging*, 17 (5), 309-325.
132. Frederiksen, K.D., Hanson, S., Hansen, S., Brixen, K., Gram, J., Jørgensen, N.R. and Støving, R.K., 2016. Bone Structural Changes and Estimated Strength After Gastric Bypass Surgery Evaluated by HR-pQCT. *Calcified Tissue International*, 98 (3), 253-262.
133. Freedson, P.S., Melanson, E. and Sirard, J., 1998. Calibration of the computer science and applications, Inc. accelerometer. *Medicine and Science in Sports and Exercice*, 30 (5), 777–781.
134. Friedl, K.E., Moore, R.J., Hoyt, R.W., Marchitelli, L.J., Martinez-Lopez, L.E. and Askew, E.W., 2000. Endocrine markers of semistarvation in healthy lean men in a multistressor environment. *Journal of Applied Physiology*, 88 (5), 1820-1830.
135. Frost, H.M. and Jee, W.S., 1992. On the rat model of human osteopenias and osteoporoses. Bone and mineral, 18 (3), 227-236.
136. Frost, H.M., 1987. The mechanostat: a proposed pathogenic mechanism of osteoporoses and the bone mass effects of mechanical and nonmechanical agents. *Bone Mineral*, 2 (2), 73-85.
137. Fukushima, N., Hanada, R., Teranishi, H., Fukue, Y., Tachibana, T., Ishikawa, H., Takeda, S., Takeuchi, Y., Fukumoto, S., Kangawa, K., Nagata, K. and Kojima, M., 2005. Ghrelin directly regulates bone formation. Journal of Bone and Mineral Research, 20 (5), 790-798.
138. Gargano, V., Massaro, M., Morra, I., Formisano, C., Di Carlo, C., and Nappi, C., 2008. Effects of two low-dose combined oral contraceptives containing drospirenone on bone turnover and bone mineral density in young fertile women: A prospective controlled randomized study. *Contraception*, 78 (1), 10-15.
139. Garnero P., 2014. New developments in biological markers of bone metabolism in osteoporosis. *Bone*, 66, 46-55.
140. Garnero, P., Sornay-Rendu, E. and Delmas, P.D., 1995. Decreased bone turnover in oral contraceptive users. *Bone*, 16 (5), 499-503.
141. Garnero, P., Vergnaud, P. and Hoyle, N., 2008. Evaluation of a fully automated serum assay for total N-terminal propeptide of type I collagen in postmenopausal osteoporosis. *Clinical Chemistry*, 54 (1), 188-196.
142. Gass, M.L., Kagan, R., Kohles, J.D., and Martens, M.G., 2008. Bone turnover marker profile in relation to the menstrual cycle of premenopausal healthy women. *Menopause*, 15 (4), 667-675.

143. Gennari, C., Agnusdei, D., Nardi, P., and Civitelli, R., 1990. Estrogen preserves a normal intestinal responsiveness to 1,25-dihydroxyvitamin D3 in oophorectomized women. *Journal Clinical Endocrinology and Metabolism*, 71, 1288–1293.
144. Gibbs, J.C., Williams, N.I. and De Souza, M.J., 2013. Prevalence of individual and combined components of the female athlete triad. *Medicine and Science in Sports and Exercise*, 45 (5), 985-996.
145. Giladi, M., Milgrom, C., Simkin, A. and Danon, Y., 1991. Stress fractures Identifiable risk factors. *The American Journal of Sports Medicine*, 19 (6), 647-652.
146. Giustina, A., Mazziotti, G., & Canalis, E., 2008. Growth hormone, insulin-like growth factors, and the skeleton. *Endocrine Reviews*, 29 (5), 535-559.
147. Glass, D.A. and Karsenty, G., 2006. Molecular bases of the regulation of bone remodeling by the canonical Wnt signaling pathway. *Current Topics in Developmental Biology*, 73, 43-84.
148. Glazer, J.L., 2008. Eating disorders among male athletes. *Current sports medicine reports*, 7 (6), 332-337.
149. Goldberg, B. and Pecora, C., 1994. Stress Fractures. *The Physician and Sportsmedicine*, 3, 68-78.
150. Golden, N. H., 2015. Assessment of Bone Health in the Young Athlete. In: The Female Athlete Triad. New York, NY: Springer.
151. Goodwin, Y., Monyeki, M.A., Boit, M.K., De Ridder, J.H. and Toriola, A.L., 2014. Association between energy availability and menstrual function in elite Kenyan runners. *African Journal for Physical, Health Education, Recreation and Dance*, 20 (2:1), 291-307.
152. Gorai, I., Taguchi, Y., Chaki, O., Nakayama, R. K. M., Yang, B. C., Yokota, S. and Minaguchi, H., 1998. Serum soluble interleukin-6 receptor and biochemical markers of bone metabolism show significant variations during the menstrual cycle. *The Journal of Clinical Endocrinology and Metabolism*, 83 (2), 326-332.
153. Gordon, C.M., Williams, A.L., Feldman, H.A., May, J., Sinclair, L., Vasquez, A. and Cox, J.E., 2008. Treatment of hypovitaminosis D in infants and toddlers. *The Journal of Clinical Endocrinology and Metabolism*, 93 (7), 2716–2721.
154. Gozansky, W.S., Van Pelt, R.E., Jankowski, C.M., Schwartz, R.S. and Kohrt, W.M., 2005. Protection of bone mass by estrogens and raloxifene during exercise-induced weight Loss. *The Journal of Clinical Endocrinology and Metabolism*, 90 (1), 52-59.
155. Greco, E.A., Fornari, R., Rossi, F., Santiemma, V., Prossomariti, G., Annoscia, C., Aversa, A., Brama, M., Marini, M., Donini, L.M. and Spera, G., 2010. Is obesity protective for osteoporosis? Evaluation of bone mineral density in individuals with high body mass index. *International Journal of Clinical Practice*, 64 (6), 817-820.
156. Greene, D.A., Naughton, G.A., Bradshaw, E., Moresi, M. and Ducher, G., 2012. Mechanical loading with or without weight-bearing activity: influence on bone strength index in elite female adolescent athletes engaged in water polo, gymnastics, and track-and-field. *Journal of Bone and Mineral Metabolism*, 30 (5), 580-587.

157. Greenleaf, C., Petrie, T.A., Carter, J. and Reel, J.J., 2009. Female collegiate athletes: prevalence of eating disorders and disordered eating behaviors. *Journal of American College Health*, 57(5), 489-496.

158. Grinspoon, S.K., Baum, H.B., Kim, V.I., Coggins, C.E. and Klibanski, A., 1995 Decreased bone formation and increased mineral dissolution during acute fasting in young women. *The Journal of Clinical Endocrinology & Metabolism,* 80 (12), 3628-3633.

159. Guerrini, M.M., Sobacchi, C., Cassani, B., Abinun, M., Kilic, S.S., Pangrazio, A., Moratto, D., Mazzolari, E., Clayton-Smith, J., Orchard, P., Coxon, F.P., Helfrich, M.H., Crockett, J.C., Mellis, D., Vellodi, A., Tezcan, I., Notarangelo, L.D., Rogers, M.J., Vezzoni, P., Villa, A. and Frattini, A., 2008. Human osteoclast-poor osteopetrosis with hypogammaglobulinemia due to TNFRSF11A (RANK) mutations. *American Journal of Human Genetics,* 83 (1), 64-76.

160. Haakonssen, E.C., Ross, M.L., Knight, E.J., Cato, L.E., Nana, A., Wluka, A.E., Cicuttini, F.M., Wang, B.H., Jenkins, D.G. and Burke, L.M., 2015. The effects of a calcium-rich pre-exercise meal on biomarkers of calcium homeostasis in competitive female cyclists: a randomised crossover trial. *PLoS One*, 10 (5), e0123302.

161. Hackney, A.C., Fahrner, C.L. and Gulledge, T.P., 1998. Basal reproductive hormonal profiles are altered in endurance trained men. *Journal of Sports Medicine and Physical Fitness*, 38 (2), 138-141.

162. Haffner, S.M. and Bauer, R.L., 1993. The association of obesity and glucose and insulin concentrations with bone density in premenopausal and postmenopausal women. *Metabolism*, 42 (6), 735-738.

163. Hagobian, T.A., Sharoff, C.G., Stephens, B.R., Wade, G.N., Silva, J.E., Chipkin, S.R. and Braun, B., 2009. Effects of exercise on energy-regulating hormones and appetite in men and women. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 296 (2), R233-R234.

164. Hagobian, T.A., Yamashiro, M., Hinkel-Lipsker, J., Streder, K., Evero, N. and Hackney, T., 2013. Effects of acute exercise on appetite hormones and ad libitum energy intake in men and women. *Applied Physiology Nutrition and Metabolism*, 38 (1), 66-72.

165. Hamrick, M.W., Ding, K.H., Ponnala, S., Ferrari, S.L. and Isales, C.M., 2008. Caloric restriction decreases cortical bone mass but spares trabecular bone in the mouse skeleton: implications for the regulation of bone mass by body weight. *Journal of Bone and Mineral Research*, 23(6), 870-878.

166. Hannon, R.A. and Eastell R., 2006. Bone markers and current laboratory assays. *Cancer Treatment Reviews*, 32 (Suppl 1), 7-14.

167. Hannon, R.A., Clowes, J.A., Eagleton, A.C., Al Hadari, A., Eastell, R. and Blumsohn, A., 2004. Clinical performance of immunoreactive tartrate-resistant acid phosphatase isoform 5b as a marker of bone resorption. *Bone*, 34 (1), 187-194.

168. Hansen M., Miller, B.F., Holm, L., Doessing, S., Petersen, S.G., Skovgaard, D., Frystyk, J., Flyvbjerg, A., Koskinen, S., Pingel, J. and Kjaer, M., 2009. Effect of administration of oral

contraceptives in vivo on collagen synthesis in tendon and muscle connective tissue in young women. *Journal of Applied Physiology*, 106 (4), 1435-1443.

169. Harris, H., 1990. The human alkaline phosphatases: what we know and what we don't know. *Clinica Chimica Acta*, 186 (2), 133-150.
170. Hartard, M., Bottermann, P., Bartenstein, P., Jeschke, D. and Schwaiger, M., 1997. Effects on bone mineral density of low-dosed oral contraceptives compared to and combined with physical activity. *Contraception*, 55 (2), 87-90.
171. Hartard, M., Kleinmond, C., Kirchbichler, A., Jeschke, D., Wiseman, M., Weissenbacher, E.R., Felsenberg, D. and Erben, R.G., 2004. Age at first oral contraceptive use as a major determinant of vertebral bone mass in female endurance athletes. *Bon*e, 35 (4), 836-841.
172. Hartard, M., Kleinmond, C., Wiseman, M., Weissenbacher, E.R., Felsenberg, D. and Erben, R.G., 2007. Detrimental effect of oral contraceptives on parameters of bone mass and geometry in a cohort of 248 young women. *Bone*, 40, (2), 444-450.
173. Harvey, N., Dennison, E. and Cooper, C., 2014. Osteoporosis: a lifecourse approach. *Journal of Bone and Mineral Research*, 29 (9), 1917-1925.
174. Hattori, S., Park, J.H., Agata, U., Akimoto, T., Oda, M., Higano, M., Aikawa, Y., Nabekura, Y., Yamato, H., Ezawa, I. and Omi, N., 2013. Influence of food restriction combined with voluntary running on bone morphology and strength in male rats. *Calcified Tissue International*, 93 (6), 540-548.
175. Hattori, S., Park, J.H., Agata, U., Oda, M., Higano, M., Aikawa, Y., Akimoto, T., Nabekura, Y., Yamato, H., Ezawa, I. and Omi, N., 2014. Food restriction causes low bone strength and microarchitectural deterioration in exercised growing male rats. *Journal of Nutritional Science and Vitaminology*, 60 (1), 35-42.
176. Hawkins J, Cifuentes M, Pleshko NL, Ambia-Sobhan H, Shapses SA., 2010. Energy restriction is associated with lower bone mineral density of the tibia and femur in lean but not obese female rats. *Journal of Nutrition*, 140 (1), 31-37.
177. Heaney, R.P. 2013 Nutrition and Risk for Osteoporosis. In: Osteoporosis, 4th Edition, Waltham, USA, Academic Press.
178. Heaney, R.P., Abrams, S., Dawson-Hughes, B., Looker, A., Looker, A., Marcus, R., Matkovic, V. and Weaver, C., 2000. Peak bone mass. *Osteoporosis international*, 11 (12), 985-1009.
179. Henriksen, D.B., Alexandersen, P., Bjarnason, N.H., Vilsbøll, T., Hartmann, B., Henriksen, E.E., Byrjalsen, I., Krarup, T., Holst, J.J. and Christiansen, C., 2003. Role of gastrointestinal hormones in postprandial reduction of bone resorption. *Journal of Bone and Mineral Research*, 18 (12), 2180-2189.
180. Henriksen, D.B., Alexandersen, P., Hartmann, B., Adrian, C.L., Byrjalsen, I., Bone, H.G., Holst, J.J. and Christiansen, C., 2007. Disassociation of bone resorption and formation by GLP-2: a 14-day study in healthy postmenopausal women. *Bone*, 40 (3), 723-729.
181. Henry, Y.M. and Eastell, R., 2000. Ethnic and gender differences in bone mineral density and bone turnover in young adults: effect of bone size. *Osteoporosis International*, 11 (6), 512-517.

182. Hernandez, C.J., 2008. How can bone turnover modify bone strength independent of bone mass? *Bone*, 42 (6), 1014-1020.

183. Hernandez, C.J., Beaupre, G.S. and Carter, D.R., 2003. A theoretical analysis of the relative influences of peak BMD, age-related bone loss and menopause on the development of osteoporosis. *Osteoporosis international*, 14 (10), 843-847.

184. Hernlund, E., Svedbom, A., Ivergård, M., Compston, J., Cooper, C., Stenmark, J., McCloskey, E.V., Jönsson, B. and Kanis, J.A., 2013. Osteoporosis in the European Union: medical management, epidemiology and economic burden. *Archives of osteoporosis*, 8 (1-2), 1-115.

185. Herrmann, M. and Herrmann, W., 2004. The assessment of bone metabolism in female elite endurance athletes by biochemical bone markers. *Clinical Chemical Laboratory Medicine*, 42 (12), 1384-1389.

186. Herrmann, M. and Seibel, M. J., 2010. The effects of hormonal contraceptives on bone turnover markers and bone health. *Clinical endocrinology*, 72 (5), 571-583.

187. Heshmati, H.M., Khosla, S., Robins, S.P., O'Fallon, W.M., Melton, L.J. and Riggs, B.L., 2002. Role of low levels of endogenous estrogen in regulation of bone resorption in late postmenopausal women. *Journal of Bone and Mineral Research*, 17 (1), 172-178.

188. Hession, M., Rolland, C., Kulkarni, U., Wise, A. and Broom, J., 2009. Systematic review of randomized controlled trials of low-carbohydrate vs. low-fat/low-calorie diets in the management of obesity and its comorbidities. *Obesity reviews*, 10 (1), 36-50.

189. Hetland, M.L., Haarbo, J. and Christiansen, C., 1993. Low bone mass and high bone turnover in male long distance runners. *The Journal of Clinical Endocrinology and Metabolism*, 77 (3), 770–775.

190. Hickey, M.S., Houmard, J.A., Considine, R.V., Tyndall, G.L., Midgette, J.B., Gavigan, K.E., Weidner, M.L., McCammon, M.R., Israel, R.G. and Caro, J.F., 1997. Gender-dependent effects of exercise training on serum leptin levels in humans. *American Journal of Physiology Endocrinology and Metabolism*, 272 (4), E562-E566.

191. Hind, K., Oldroyd, B. and Truscott, J.G., 2006. In vivo precision of the GE Lunar iDXA densitometer for the measurement of total body composition and fat distribution in adults. *European Journal of Clinical Nutrition*, 65 (1), 140-142.

192. Hlaing, T.T. and Compston, J.E., 2014. Biochemical markers of bone turnover–uses and limitations. *Annals of Clinical Biochemistry*, 51 (Pt 2), 189-202.

193. Hoch, A.Z., Pajewski, N.M., Moraski, L., Carrera, G.F., Wilson, C.R., Hoffmann, R.G., Schimke, J.E. and Gutterman, D.D., 2009. Prevalence of the female athlete triad in high school athletes and sedentary students. Clinical journal of sport medicine: official journal of the Canadian Academy of Sport Medicine, 19(5), 421.

194. Holick, M.F., Binkley, N.C., Bischoff-Ferrari, H.A., Gordon, C.M., Hanley, D.A., Heaney, R.P., Murad, M.H. and Weaver, C.M., 2011. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. *The Journal of Clinical Endocrinology & Metabolism*, 96 (7), 1911-1930.

195. Horsman, A., Jones, M., Francis, R. and Nordin, C., 1983. The effect of estrogen dose on postmenopausal bone loss. *New England Journal of Medicine*, 309 (23), 1405-1407.
196. Howley, E.T., Bassett, D.R. and Welch, H.G., 1995. Criteria for maximal oxygen uptake: review and commentary. *Medicine and Science in Sports and Exercise*, 27 (9), 1292-1301.
197. Hoyt, R.W. and Friedl, K.E., 2006. Field studies of exercise and food deprivation. *Current Opinion in Clinical Nutrition and Metabolic Care*, 9 (6), 685-690.
198. Hsin, M.C., Huang, C.K., Tai, C.M., Yeh, L.R., Kuo, H.C. and Garg, A., 2015 A case-matched study of the differences in bone mineral density 1 year after 3 different bariatric procedures. *Surgery for Obesity and Related Disorders*, 11 (1), 181-185.
199. Huang, B.K., Golden, L.A., Tarjan, G., Madison, L.D. and Stern, P.H., 2000. Insulin-like growth factor I production is essential for anabolic effects of thyroid hormone in osteoblasts. *Journal of Bone and Miner Research*, 15 (2), 188–197.
200. Huda, M.S.B., Durham, B.H., Wong, S.P., Dovey, T.M,, McCullochm, P., Kerrigan, D., Pinkney, J.H., Fraser, W.D. and Wilding, J.P.H., 2007. Lack of an acute effect of ghrelin on markers of bone turnover in healthy controls and post-gastrectomy subjects. *Bone*, 41 (3), 406-413.
201. Hughes, J.M., Smith, M.A., Henning, P.C., Scofield, D.E., Spiering, B.A., Staab, J.S., Hydren, J.R., Nindl,, B.C. and Matheny, J.R.W., 2014. Bone formation is suppressed with multi-stressor military training. *European journal of applied physiology*, 114 (11), 2251-2259.
202. Hwang, Y.C., Jeong, I.K., Ahn, K.J. and Chung, H.Y., 2012. Circulating osteocalcin level is associated with improved glucose tolerance, insulin secretion and sensitivity independent of the plasma adiponectin level. *Osteoporosis International*, 23 (4), 1337-1342.
203. Ihle, R. and Loucks, A.B., 2004. Dose-response relationships between energy availability and bone turnover in young exercising women. *Journal of Bone and Mineral Research*, 19 (8), 1231–1240.
204. Ilich, J.Z. and Kerstetter, J.E., 2000. Nutrition in bone health revisited: a story beyond calcium. *Journal of American College of Nutrition*, 19 (6), 715-737.
205. IPAQ, 2005. Guidelines for Data Processing and Analysis of the International Physical Activity Questionnaire (IPAQ) - Short Form and Long Forms. Retrieved from https://sites.google.com/site/theipaq
206. Iwamoto, J. and Takeda, T., 2003. Stress fractures in athletes: review of 196 cases. *Journal of Orthopaedic Science*, 8 (3), 273-278.
207. Iwaniec, U.T. and Turner, R.T., 2016. Influence of body weight on bone mass, architecture, and turnover. *Journal of Endocrinology*, 230 (3), R115-R130.
208. Jenkins, N., Black, M., Paul, E., Pasco, J.A., Kotowicz, M.A. snd Schneider, H.G,, 2013. Age-related reference intervals for bone turnover markers from an Australian reference population. *Bone*, 55 (2), 271-276.
209. Jiménez-Reina, L., Maartens, P. J., Jimena-Medina, I., Agarwal, A. and du Plessis, S. S., 2016. Overview of the Male Reproductive System. In: Exercise and Human Reproduction, New York, NY: Springer.

210. Johnell, O. and Kanis, J.A., 2006. An estimate of the worldwide prevalence and disability associated with osteoporotic fractures. *Osteoporosis international*, 17 (12), 1726-1733.

211. Johnson , L., May, M., Busbee, D. and Williams, J., 1992. Effect of age and dietary restriction on daily sperm production and number and transit time of epididymal spermatozoa in the mouse. *Age*. 15 (3), 65-71.

212. Johnson, A.W., Weiss, C.B. and Wheeler, D.L., 1994. Stress fractures of the femoral shaft in athletes-more common than expected a new clinical test. *The American Journal of Sports Medicine*, 22 (2), 248-256.

213. Jones, A.M. and Doust, J.H., 1996. A 1% treadmill grade most accurately reflects the energetic cost of outdoor running. *Journal of Sports Sciences*, 14 (4), 321-327.

214. Jones, B.H., Thacker, S.B., Gilchrist, J., Kimsey, C.D. and Sosin, D.M., 2002 Prevention of lower extremity stress fractures in athletes and soldiers: a systematic review. *Epidemiologic Reviews*, 24 (2), 228-247.

215. Joy, E., Kussman, A. and Nattiv, A., 2016. 2016 update on eating disorders in athletes: A comprehensive narrative review with a focus on clinical assessment and management. *British Journal of Sports Medicine*, 50 (3), 154-162.

216. Julian-Almarcegui, C., Gomez-Cabello, A., Gonzalez-Agüero, A., Olmedillas, H., Gomez-Bruton, A., Matute-Llorente, A., Casajús, J.A. and Vicente-Rodríguez, G., 2013. The nutritional status in adolescent Spanish cyclists. Nutricion Hospitalaria, 28 (4), 1184-1189.

217. Kanis, J.A., 1994. Assessment of fracture risk and its application to screening for postmenopausal osteoporosis: synopsis of a WHO report. *Osteoporosis International*, 4 (6), 368-381.

218. Kanis, J.A., McCloskey, E.V., Johansson, H., Cooper, C., Rizzoli, R. and Reginster, J.Y., 2013. European guidance for the diagnosis and management of osteoporosis in postmenopausal women. *Osteoporosis international*, 24 (1), 23-57.

219. Kannus, P., Haapasalo, H., Sankelo, M., Sievanen, H., Pasanen, M., Heinonen, A., Oja, P. and Vuori, I., 1995. Effect of starting age of physical activity on bone mass in the dominant arm of tennis and squash players. *Annals of Internal Medicine*, 123 (1), 27-31.

220. Karsenty, G. and Ferron, M., 2012. The contribution of bone to whole-organism physiology. *Nature*, 481 (7381), 314-320.

221. Kassanos, D., Trakakis, E., Baltas, C.S., Papakonstantinou, O., Simeonidis, G., Salamalekis, G., Grammatikakis, I., Basios, G., Labos, G., Skarantavos, G. and Balanika. A., 2010. Augmentation of cortical bone mineral density in women with polycystic ovary syndrome: a peripheral quantitative computed tomography (pQCT) study. *Human reproduction*, 25 (8), 2107-2114.

222. Katzeff, H.L. and Selgrad, C., 1991. Maintenance of thyroid hormone production during exercise-induced weight loss. *American Journal of Physiology*, 261 (3 Pt 1), E382-E388.

223. Katzeff, H.L., Bovbjerg, D. and Mark, D.A., 1988. Exercise regulation of triiodothyronine metabolism. *American Journal of Physiology*, 255 (6 Pt 1), E824-E828.

224. Kelly, E.W., 2000. Stress fractures of the pelvis in female Navy recruits: an analysis of possible mechanisms of injury. *Military medicine*, 165 (2), 142-146.

225. Kemmler, W., Engelke, K., Weineck, J., Hensen, J. and Kalender, W.A., 2003. The Erlangen Fitness Osteoporosis Prevention Study: a controlled exercise trial in early postmenopausal women with low bone density-first-year results. *Archives of Physical Medicine and Rehabilation*, 84 (5), 673-82.

226. Kerstetter, J.E., Kenny, A.M. and Insogna, K.L., 2011. Dietary protein and skeletal health: a review of recent human research. *Current opinion in Lipidology*, 22 (1), 16.

227. Khosla, S. and Pacifici, R., 2013. Estrogen Deficiency, Postmenopausal Osteoporosis, and Age-Related Bone Loss. In: Osteoporosis, 4th Edition, Waltham: Academic Press.

228. Khosla, S., Atkinson, E.J., Melton, L.J., Riggs, B.L., 1997. Effects of age and estrogen status on serum parathyroid hormone levels and biochemical markers of bone turnover in women: a population-based study. *The Journal of Clinical Endocrinology and Metabolism*, 82 (5), 1522-1527.

229. Khosla, S., Melton III, L.J., Atkinson, E.J., O'fallon, W.M., Klee, G.G. and Riggs, B.L., 1998. Relationship of serum sex steroid levels and bone turnover markers with bone mineral density in men and women: a key role for bioavailable estrogen 1. *The Journal of Clinical Endocrinology and Metabolism*, 83 (7), 2266-2274.

230. Khosla, S., Oursler, M.J. and Monroe, D.G., 2012. Estrogen and the skeleton. *Trends Endocrinology and Metabolism*, 23 (11), 576-581.

231. Kimber, N.E., Ross, J.J., Mason, S.L. and Speedy, D.B., 2002. Energy balance during an ironman triathlon in male and female triathletes. *International journal of sport nutrition and exercise metabolism*, 12, 47-62.

232. Kindblom, J.M., Gevers, E.F., Skrtic, S.M., Lindberg, M.K., Göthe, S., Törnell, J., Vennström, B. and Ohlsson, C., 2005. Increased adipogenesis in bone marrow but decreased bone mineral density in mice devoid of thyroid hormone receptors. *Bone*, 36 (4), 607-616.

233. King, J.A., Wasse, L.K., Ewens, J., Crystallis, K., Emmanuel, J., Batterham, R.L. and Stensel D.J., 2011. Differential acylated ghrelin, peptide YY3-36, appetite, and food intake responses to equivalent energy deficits created by exercise and food restriction. *The Journal of Clinical Endocrinology and Metabolism*, 96 (4), 1114-1121.

234. King, N.A., Hopkins, M., Caudwell, P., Stubbs, R.J. and Blundell, J.E., 2008. Individual variability following 12 weeks of supervised exercise: identification and characterization of compensation for exercise-induced weight loss. *International Journal of Obesity*, 32(1), 177-184.

235. King, N.A., Horner, K., Hills, A.P., Byrne, N.M., Wood, R.E., Bryant, E., Caudwell, P., Finlayson, G., Gibbons, C., Hopkins, M., Martins, C. and Blundell, J.E., 2012. Exercise, appetite and weight management: understanding the compensatory responses in eating behaviour and how they contribute to variability in exercise-induced weight loss. *British Journal of Sports Medicine*, 46 (5), 315-322.

236. Klibanski, A., Biller BM, Schoenfeld DA, Herzog DB and Saxe VC, 1995. The effects of estrogen administration on trabecular bone loss in young women with anorexia nervosa. *The Journal of Clinical Endocrinology and Metabolism*, 80 (3), 898-904.

237. Klotzbuecher, C.M., Ross, P.D., Landsman, P.B., Abbott, T.A. and Berger, M., 2000. Patients with prior fractures have an increased risk of future fractures: a summary of the literature and statistical synthesis. *Journal of Bone and Mineral Research*, 15 (4), 721-739.
238. Koehler, K., Hoerner, N.R., Gibbs, J.C., Zinner, C., Braun, H., De Souza, M.J. and Schaenzer, W., 2016. Low energy availability in exercising men is associated with reduced leptin and insulin but not with changes in other metabolic hormones. *Journal of Sports Sciences*, 34 (20), 1921-1929.
239. Kojima, M. and Kangawa, K., 2005. Ghrelin: structure and function. *Physiological reviews*, 85 (2), 495-522.
240. Kontulainen, S., Kannus, P., Haapasalo, H., Sievänen, H., Pasanen, M., Heinonen, A., Oja, P. and Vuori, I., 2001. Good maintenance of exercise-induced bone gain with decreased training of female tennis and squash players: a prospective 5-year follow-up study of young and old starters and controls. *Journal of Bone and Mineral Research*, 16 (2), 195-201.
241. Kular, J., Tickner, J., Chim, S.M. and Xu, J., 2012. An overview of the regulation of bone remodelling at the cellular level. *Clinical Biochemistry*, 45 (12), 863-873.
242. Kussman, A., Fredericson, M., Kraus, E., Kim, B., Tenforde, A. and Nattiv, A., 2015. Risk factors for bone stress injuries in NCAA division I collegiate distance runners: a 3 year retrospective study. *Clinical Journal of Sports Medicine*, 25 (2), 195.
243. Kyriazis, G.A., Caplan, J.D., Lowndes, J., Carpenter, R.L., Dennis, K.E., Sivo, S.A. and Angelopoulos, T.J., 2007. Moderate exercise-induced energy expenditure does not alter leptin levels in sedentary obese men. *Clinical Journal of Sports Medicine*, 17 (1), 49-51.
244. Kyrolainen, H., Karinkanta, J., Santtila, M., Koski, H., Mantysaari, M. and Pullinen, T., 2008. Hormonal responses during a prolonged military field exercise with variable exercise intensity. *European Journal of Applied Physiology*, 102 (5), 539–546.
245. Lader, D., 2009. Opinions survey report no 41. Contraception and sexual health, 2008-9. Office for National Statistics, 2009. Retrieved from www.ons.gov.uk/ons/rel/lifestyles/contraception-and-sexualhealth/2008-09/index.html.
246. Lakatos, P., 2003. Thyroid hormones: beneficial or deleterious for *bone? Calcified Tissue International*, 73 (3), 205-209.
247. Lakatos, P., Foldes, J., Nagy, Z., Takacs, I., Speer, G., Horvath, C., Mohan, S., Baylink, D.J. and Stern, P.H., 2000. Serum insulin-like growth factor-I, insulin-like growth factor binding proteins, and bone mineral content in hyperthyroidism. *Thyroid*, 10 (5), 417–423.
248. Lanyon, L.E, Armstrong, V., Ong, D., Zaman, G. and Price, J., 2004. Is estrogen receptor alpha key to controlling bones' resistance to fracture? *Journal of Endocrinology*, 182(2), 183-191.
249. Lanyon, L.E., 1996. Using functional loading to influence bone mass and architecture: objectives, mechanisms, and relationship with estrogen of the mechanically adaptive process in bone. *Bone*, 18(1 Suppl), 37S-43S.
250. Lappe, J., Cullen, D., Haynatzki, G., Recker, R., Ahlf, R. and Thompson, K., 2008. Calcium and vitamin D supplementation decreases incidence of stress fractures in female Navy recruits. *Journal of Bone Mineral Research*, 23 (5), 741-749.

251. Lawson, E.A., Donoho, D., Miller, K.K., Misra, M., Meenaghan, E., Lydecker, J., Wexler, T., Herzog, D.B. and Klibanski, A., 2009. Hypercortisolemia is associated with severity of bone loss and depression in hypothalamic amenorrhea and anorexia nervosa. *The Journal of Clinical Endocrinology and Metabolism*, 94 (12), 4710-4716.

252. Lawson, E.A., Miller, K.K., Bredella, M.A., Phan, C., Misra, M., Meenaghan, E., Rosenblum, L., Donoho, D., Gupta, R. and Klibanski, A., 2010. Hormone predictors of abnormal bone microarchitecture in women with anorexia nervosa. *Bone*, 46 (2), 458-463.

253. Leder, B.Z., LeBlanc, K.M., Schoenfeld, D.A., Eastell, R. and Finkelstein, J.S., 2003. Differential effects of androgens and estrogens on bone turnover in normal men. *The Journal of Clinical Endocrinology and Metabolism*, 88 (1), 204-210.

254. Lee, A., Hodges, S. and Eastell, R., 2000. Measurement of osteocalcin. *Annals of Clinical Biochemistry*, 37, 432–446.

255. Lee, K. and Lanyon, L.E., 2004. Mechanical loading influences bone mass through estrogen receptor α. *Exercise and Sport Sciences Reviews*, 32 (2), 64-68.

256. Leidy, H.J., Carnell, N.S., Mattes, R.D. and Campbell, W.W., 2007. Higher protein intake preserves lean mass and satiety with weight loss in pre-obese and obese women. *Obesity (Silver Spring)*, 15 (2), 421–429.

257. Levine J.A., 2005. Measurement of energy expenditure. Public Health Nutrition, 8(7A), 1123-32.

258. Lewis, R.M., Redzic, M. and Thomas, D.T., 2013. The effects of season-long vitamin D supplementation on collegiate swimmers and divers. International journal of sport nutrition and exercise metabolism, 23 (5), 431-440.

259. Li, F. and Mühlbauer, R.C., 1999. Food fractionation is a powerful tool to increase bone mass in growing rats and to decrease bone loss in aged rats: modulation of the effect by dietary phosphate. *Journal of Bone and Mineral Research*, 14 (8), 1457-1465.

260. Li, X., Zhang, Y., Kang, H., Liu, W., Liu, P., Zhang, J. and Harris, S.E. and Wu, D., 2005. Sclerostin binds to LRP5/6 and antagonizes canonical Wnt signalling. *Journal of Biological Chemistry*, 280 (20) 19883-19887.

261. Link, T.M., Vieth, V., Stehling, C., Lotter, A., Beer, A., Newitt, D. and Majumdar, S., 2003. High-resolution MRI vs multislice spiral CT: which technique depicts the trabecular bone structure best? European Radiology, 13 (4), 663- 671.

262. Liu, J.M., Zhao, H.Y., Ning, G., Chen, Y., Zhang, L.Z., Sun, L.H., Zhao, Y.J., Xu, M.Y. and Chen, J.L.,2008. IGF-1 as an early marker for low bone mass or osteoporosis in premenopausal and postmenopausal women. *Journal of Bone and Mineral Metabolism*, 26 (2), 159-164.

263. Liu, S.L. and Lebrun, C.M., 2006. Effect of oral contraceptives and hormone replacement therapy on bone mineral density in premenopausal and perimenopausal women: a systematic review. *British Journal Sports Medicine*, 40 (1), 11-24.

264. Löfgren, B., Detter, F., Dencker, M., Stenevi-Lundgren, S., Nilsson, J.Å. and Karlsson, M.K., 2011. Influence of a 3-year exercise intervention program on fracture risk, bone mass, and bone size in prepubertal children. *Journal of Bone and Mineral Research*, 26 (8), 1740-1747.

265. Lombardi, G., Lanteri, P., Colombini, A. and Banfi, G., 2012a. Blood biochemical markers of bone turnover: pre-analytical and technical aspects of sample collection and handling. *Clinical Chemistry Laboratory Medicine* 50 (5), 771-789.
266. Lombardi, G., Lanteri, P., Graziani, R., Colombini, A., Banfi, G. and Corsetti, R., 2012b. Bone and energy metabolism parameters in professional cyclists during the Giro d'Italia 3-weeks stage race. *PloS One*, 7 (7), e42077.
267. Lombardi, G., Perego, S., Luzi, L. and Banfi, G., 2015b. A four-season molecule: osteocalcin. Updates in its physiological roles. *Endocrine*, 48 (2), 394-404.
268. Lombardi, G., Sanchis-Gomar, F., Perego, S., Sansoni, V. and Banfi, G., 2015a. Implications of exercise-induced adipo-myokines in bone metabolism. *Endocrine*, 1-22.
269. Lorentzon, M., Swanson, C., Andersson, N., Mellstrom, D. and Ohlsson, C., 2005. Free Testosterone is a Positive Whereas Free Estradiol is a Negative Predictor of Cotical Bone Size in Young Swedish Men: The GOOD Study. *Journal of Bone and Mineral Research*, 20 (8), 1334-1340.
270. Loucks, A.B. and Callister, R., 1993. Induction and prevention of low-T3 syndrome in exercising women. *American Journal of Physiology*, 264 (5 Pt 2), R924–R930.
271. Loucks, A.B. and Heath, E.M., 1994. Induction of low-T3 syndrome in exercising women occurs at a threshold of energy availability. *American Journal of Physiology*. 1994, 266(3Pt 2), R817–R823.
272. Loucks, A.B. and Thuma, J.R., 2003. Luteinizing hormone pulsatility is disrupted at a threshold of energy availability in regularly menstruating women. *The Journal of Clinical Endocrinology and Metabolism*, 88 (1), 297-311.
273. Loucks, A.B., 2007. Low energy availability in the marathon and other endurance sports. *Sports Medicine*, 37 (4-5), 348-352.
274. Loucks, A.B., 2013. Energy balance and energy availability. In: The Encyclopaedia of Sports Medicine: An IOC Medical Commission Publication, Volume 19, Chichester, UK, John Wiley & Sons Ltd.
275. Loucks, A.B., Kiens, B. and Wright, H.H., 2011. Energy availability in athletes. *Journal of Sports Sciences*, 29 (Suppl 1), S7–S15.
276. Loucks, A.B., Verdun, M. and Heath, E.M., 1998. Low energy availability, not stress of exercise, alters LH pulsatility in exercising women. *Journal of Applied Physiology (1985)*, 84 (1), 37-46.
277. Lucía, A., Chicharro, J.L., Pérez, M., Serratosa, L., Bandrés, F. and Legido, J.C., 1996. Reproductive function in male endurance athletes: sperm analysis and hormonal profile. *Journal of Applied Physiology*, 81 (6), 2627-2636.
278. Lüftner, D., Jozereau, D., Schildhauer, S., Geppert, R., Müller, C., Fiolka, G., Wernecke, K.D. and Possinger, K., 2005. PINP as serum marker of metastatic spread to the bone in breast cancer patients. *Anticancer Research*, 25 (3A), 1491-1499.

279. Lutz, L.J., Karl, J.P., Rood, J.C., Cable, S.J., Williams, K.W., Young, A.J. and McClung, J.P., 2012. Vitamin D status, dietary intake, and bone turnover in female Soldiers during military training: a longitudinal study. *Journal of the International Society of Sports Nutrition*, 9 (1), 38.

280. Macdonald H.M, Christa, L. and McKay H.A., 2013 Bone Acquisition in Adolescence. In: Osteoporosis, 4th Edition, Waltham, Academic Press.

281. MacDougall, J.D., Webber, C.E., Martin, J., Ormerod, S., Chesley, A., Younglai, E.V., Gordon, C.L., Blimkie, C.J., 1992. Relationship among running mileage, bone density, and serum testosterone in male runners. *Journal of Applied Physiology*, 73 (3), 1165-1170.

282. MacNeil, J.A. and Boyd, S.K., 2008. Improved reproducibility of high-resolution peripheral quantitative computed tomography for measurement of bone quality. *Medical Engineering and Physics*, 30 (6), 792-799.

283. Magkos, F. and Yannakoulia, M., 2003. Methodology of dietary assessment in athletes: concepts and pitfalls. *Current Opinion in Clinical Nutrition & Metabolic Care*, 6 (5), 539-549.

284. Maguire, K. and Westhoff, C., 2011. The state of hormonal contraception today: established and emerging noncontraceptive health benefits. *American Journal of Obstetrics and Gynecology*, 205 (4 Suppl.), S4–S8.

285. Maïmoun, L., Georgopoulos, N.A. and Sultan, C., 2014. Endocrine disorders in adolescent and young female athletes: impact on growth, menstrual cycles, and bone mass acquisition. *The Journal of Clinical Endocrinology and Metabolism*, 99 (11), 4037-4050.

286. Maïmoun, L., Lumbroso, S., Manetta, J., Paris, F., Leroux, J.L. and Sultan C, 2003. Testosterone is significantly reduced in endurance athletes without impact on bone mineral density. *Hormone Research*, 59 (6), 285-292.

287. Maïmoun, L., Mura, T., Leprieur, E., Avignon, A., Mariano-Goulart, D. and Sultan, A., 2016. Impact of obesity on bone mass throughout adult life: Influence of gender and severity of obesity. *Bone*, 90, 23-30.

288. Mallinson, R.J. and De Souza, M.J., 2014. Current perspectives on the etiology and manifestation of the "silent" component of the Female Athlete Triad. *International Journal of Women's Health*, 3 (6), 451-467.

289. Manolagas, S.C., Kousteni, S. and Jilka, R.L., 2002. Sex steroids and bone. *Recent Progress in Hormone Research*, 57, 385-409.

290. Manolagas, S.C., O'Brien, C.A. and Almeida, M., 2013. The role of estrogen and androgen receptors in bone health and disease. *Nature Reviews Endocrinology*, 9 (12), 699-712.

291. Manske, S.L., Macdonald, H.M., Nishiyama, K.K., Boyd, S.K. and McKay, H.A., 2010. Clinical tools to evaluate bone strength. *Clinical Reviews in Bone and Mineral Metabolism*, 8 (3), 122-134.

292. Marques, E.A., Mota, J., Machado, L., Sousa, F., Coelho, M., Moreira, P. and Carvalho, J., 2011. Multicomponent training program with weight-bearing exercises elicits favorable bone density, muscle strength, and balance adaptations in older women. *Calcified tissue international*, 88(2), 117-129.

293. Marshall, D., Johnell, O. and Wedel, H., 1996. Meta-analysis of how well measures of bone mineral density predict occurrence of osteoporotic fractures. BMJ, 312 (7041), 1254-1259.

294. Martin, B., Pearson, M., Kebejian, L., Golden, E., Keselman, A., Bender, M., Carlson, O., Egan, J., Ladenheim, B., Cadet, J.L. and Becker, K.G., 2007. Sex-dependent metabolic, neuroendocrine and cognitive responses to dietary energy restriction and excess. *Endocrinology*, 148 (9), 4318-4333.

295. Martin, T.J. and Seeman, E., 2008. Bone remodelling: its local regulation and the emergence of bone fragility. *Best Practice and Research in Clinical Endocrinology and Metabolism*, 22 (5), 701-722.

296. Matheson, G.O., Clement, D.B., McKenzie, D.C., Taunton, J.E., Lloyd-Smith, D.R. and MacIntyre, J.G., 1987. Stress fractures in athletes. A study of 320 cases. A*merican Journal of Sports Medicine*, 15 (1), 46-58.

297. Matkovic, V. and Heaney, R.P., 1992. Calcium balance during human growth: evidence for threshold behavior. *The American Journal of Clinical Nutrition*, 55 (5), 992-996.

298. Matkovic, V., 1992. Calcium and peak bone mass. *Journal of internal medicine*, 231 (2), 151-160.

299. Matthews, C.E., Ainsworth, B.E., Thompson, R.W., & Bassett D.R., 2002. Sources of variance in daily physical activity levels as measured by an accelerometer. *Medicine and Science in Sports and Exercise*, 34 (8), 1376-1381.

300. Mazzetti, S., Wolff, C., Yocum, A., Reidy, P., Douglass, M., Cochran, M. and Douglass, M., 2011. Effect of maximal and slow versus recreational muscle contractions on energy expenditure in trained and untrained men. *The Journal of Sports Medicine and Physical Fitness*, 51(3), 381-392.

301. Mazziotti, G., Chiavistelli, S. and Giustina, A., 2015. Pituitary diseases and bone. *Endocrinology Metabolism Clinics of North America*, 44 (1), 171-180.

302. McKane, W.R., Khosla, S., Burritt, M.F., Kao, P.C., Wilson, D.M., Ory, S.J. and Riggs, B.L., 1995. Mechanism of renal calcium conservation with estrogen replacement therapy in women in early postmenopause–a clinical research center study. Journal of Clinical Endocrinology and Metabolism. 80 (12), 3458–3464.

303. McClung, J.P. and Karl, J.P., 2010. Vitamin D and stress fracture: the contribution of vitamin D receptor gene polymorphisms. *Nutrition Reviews*, 68, 365-369.

304. Melin, A., Tornberg, Å.B., Skouby, S., Møller, S.S., Faber, J., Sundgot-Borgen, J. and Sjödin, A., 2016. Low-energy density and high fiber intake are dietary concerns in female endurance athletes. *Scandinavian Journal of Medicine and Science in Sports*, 26 (9), 1060-1071.

305. Melin, A., Tornberg, Å.B., Skouby, S., Møller, S.S., Sundgot-Borgen, J., Faber, J., Sidelmann, J.J., Aziz, M. and Sjödin, A., 2015. Energy availability and the female athlete triad in elite endurance athletes. Scandinavian Journal of Medicine and Science in Sports, 25 (5), 610-622.

306. Metzger, C.E., Baek, K., Swift, S.N., De Souza, M.J. and Bloomfield, S.A., 2016. Exercise during energy restriction mitigates bone loss but not alterations in estrogen status or metabolic hormones. *Osteoporosis International*, 1-10.

307. Meyer, U., Romann, M., Zahner, L., Schindler, C., Puder, J.J., Kraenzlin, M., Rizzoli, R. and Kriemler, S., 2011. Effect of a general school-based physical activity intervention on bone mineral content and density: a cluster-randomized controlled trial. *Bone*, 48 (4), 792-797.
308. Midgley, A.W. and Carroll, S., 2009. Emergence of the verification phase procedure for confirming 'true'VO2max. *Scandinavian journal of medicine & science in sports*, *19*(3), 313-322.
309. Milgrom, C., Finestone, A., Segev, S., Olin, C., Arndt, T. and Ekenman, I., 2003. Are overground or treadmill runners more likely to sustain tibial stress fracture? *British journal of sports medicine*, 37(2), 160-163.
310. Milgrom, C., Giladi, M., Kashtan, H., Simkin, A., Chisin, R., Margulies, J., Steinberg, R., Aharonson, Z. and Stein, M., 1985. A prospective study of the effect of a shock-absorbing orthotic device on the incidence of stress fractures in military recruits. *Foot and Ankle International*, 6 (2), 101-104.
311. Miller, G.D., Jarvis, J.K. and McBean, L.D., 2001. The Importance of Meeting Calcium Needs with Foods. The Journal of the American College of Nutrition, 20 (2), 168S-185S.
312. Miller, K.K., 2011. Endocrine dysregulation in anorexia nervosa update. *The Journal of Clinical Endocrinology and Metabolism*, 96 (10), 2939-2949.
313. Milligan, G.S., Reilly, T.J., Zumbo, B.D. and Tipton, M.J., 2016. Validity and reliability of physical employment standards. *Applied Physiology, Nutrition, and Metabolism*, 41(6), S83-S91.
314. Misra, M. and Klibanski, A., 2011. The neuroendocrine basis of anorexia nervosa and its impact on bone metabolism. *Neuroendocrinology*, 93 (2), 65-73.
315. Misra, M. and Klibanski, A., 2014. Anorexia nervosa and bone. *Journal of Endocrinology*, 221 (3), R163-R176.
316. Misra, M., 2012. Effects of hypogonadism on bone metabolism in female adolescents and young adults. *Nature Reviews Endocrinology*, 8 (7), 395-404.
317. Misra, M., Aggarwal, A., Miller, K.K., Almazan, C., Worley, M., Soyka, L.A., Herzog, D.B. and Klibanski, A., 2004. Effects of anorexia nervosa on clinical, hematologic, biochemical, and bone density parameters in community-dwelling adolescent girls. *Pediatrics*, 114 (6), 1574-1583.
318. Misra, M., Miller, K.K., Cord, J., Prabhakaran, R., Herzog, D.B., Goldstein, M., Katzman, D.K. and Klibanski, A., 2007. Relationships between serum adipokines, insulin levels, and bone density in girls with anorexia nervosa. *The Journal of Clinical Endocrinology and Metabolism*, 92 (6), 2046-2052.
319. Misra, M., Prabhakaran, R., Miller, K.K., Goldstein, M.A., Mickley, D., Clauss, L., Lockhart, P., Cord, J., Herzog, D.B., Katzman, D.K. and Klibanski, A., 2008. Weight gain and restoration of menses as predictors of bone mineral density change in adolescent girls with anorexia nervosa-1. *The Journal of Clinical Endocrinology and Metabolism*, 93 (4), 1231-1237.
320. Mitchell, D.M., Tuck, P., Ackerman, K.E., Sokoloff, N.C., Woolley, R., Slattery, M., Lee, H., Bouxsein, M.L. and Misra, M., 2015. Altered trabecular bone morphology in adolescent and young adult athletes with menstrual dysfunction. *Bone*, 81, 24-30.

321. Mittendorfer, B., Horowitz, J.F. and Klein, S., 2001. Gender differences in lipid and glucose kinetics during short-term fasting. *American Journal of Physiology Endocrinology and Metabolism*, 281 (6), E1333-E1339.
322. Mochizuki, H., Hakeda, Y., Wakatsuki, N., Usui, N.O., Akashi, S.H., Sato, T.A., Tanaka, K.A. and Kumegawa, M.A., 1992. Insulin-like growth factor-I supports formation and activation of osteoclasts. *Endocrinology*, 131 (3), 1075-1080.
323. Mohr, M., Helge, E.W., Petersen, L.F., Lindenskov, A., Weihe, P., Mortensen, J., Jørgensen, N.R. and Krustrup, P., 2015. Effects of soccer vs swim training on bone formation in sedentary middle-aged women. *European Journal of Applied Physiology*, 115 (12), 2671-2679.
324. Morgan, E.F., Barnes, G.L., and Einhorn, T.A., 2013. The bone organ system: form and function. In: Osteoporosis, 4th Edition, Waltham, Academic Press.
325. Morgan, J.F., Reid, F. and Lacey, H.J, 1999. The SCOFF questionnaire: assessment of a new screening tool for eating disorders. *British Medical Journal*, 319 (7223), 1467–1468.
326. Mountjoy, M., Sundgot-Borgen, J., Burke, L., Carter, S., Constantini, N., Lebrun, C., Meyer, N., Sherman, R., Steffen, K., Budgett, R. and Ljungqvist, A., 2014. The IOC consensus statement: beyond the Female Athlete Triad--Relative Energy Deficiency in Sport (RED-S). *British Journal of Sports Medicine*, 48 (7), 491-497.
327. Mountjoy, M., Sundgot-Borgen, J., Burke, L., Carter, S., Constantini, N., Lebrun, C., Meyer, N., Sherman, R., Steffen, K., Budgett, R. and Ljungqvist, A., 2015. Authors' 2015 additions to the IOC consensus statement: Relative Energy Deficiency in Sport (RED-S). *British Journal of Sports Medicine*, 49 (7), 417-420.
328. Mundy, G.R., and Guise, T.A., 1999. Hormonal control of calcium homeostasis. *Clinical chemistry*, 45 (8), 1347-1352.
329. Nakamura, K., Nashimoto, M., Matsuyama, S. and Yamamoto, M., 2001. Low serum concentrations of 25-hydroxyvitamin D in young adult Japanese women: a cross sectional study. *Nutrition*, 17 (11), 921-925.
330. Nakashima, K., Zhou, X., Kunkel, G., Zhang, Z., Deng, J.M., Behringer, R.R., de Crombrugghe, B., 2002. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell*, 108 (1), 17-29.
331. Nakata, Y., Ohkawara, K., Lee, D.J., Okura, T. and Tanaka, K., 2008. Effects of additional resistance training during diet-induced weight loss on bone mineral density in overweight premenopausal women. *Journal of Bone and Mineral Metabolism*, 26 (2), 172-177.
332. Nana, A., Slater, G.J., Stewart, A.D. and Burke, L.M., 2015. Methodology review: using dual-energy X-ray absorptiometry (DXA) for the assessment of body composition in athletes and active people. *International Journal of Sport Nutrition and Exercise Metabolism*, 25 (2), 198-215.
333. Naot, D. and Cornish, J., 2014. Cytokines and hormones that contribute to the positive association between fat and bone. *Frontiers in endocrinology*, 5, 70.
334. Nappi, C., Bifulco, G., Tommaselli, G.A., Gargano, V. and Di Carlo, C., 2012. Hormonal contraception and bone metabolism: a systematic review. *Contraception*, 86 (6), 606-621.

335. Nappi, C., Di Spiezio Sardo, A., Acunzo, G., Bifulco, G., Tommaselli, G.A., Guida, M. and Di Carlo, C., 2003. Effects of a low-dose and ultra-low-dose combined oral contraceptive use on bone turnover and bone mineral density in young fertile women: a prospective controlled randomized study. *Contraception*, 67 (5), 355-359.

336. Nass, R., Pezzoli, S.S., Oliveri, M.C., Patrie, J.T., Harrell, F.E., Clasey, J.L., Heymsfield, S.B., Bach, M.A., Vance, M.L. and Thorner, M.O., 2008. Effects of an oral ghrelin mimetic on body composition and clinical outcomes in healthy older adults: a randomized trial. *Annals of Internal Medicine*, 149 (9), 601-611.

337. Nattiv, A., Kennedy, G., Barrack, M.T., Abdelkerim, A., Goolsby, M.A., Arends, J.C. and Seeger, L.L., 2013. Correlation of MRI grading of bone stress injuries with clinical risk factors and return to play a 5-year prospective study in collegiate track and field athletes. *American Journal of Sports Medicine*, 41 (8), 1930-1941.

338. Nattiv, A., Loucks, A.B., Manore, M.M., Sanborn, C.F., Sundgot-Borgen, J. and Warren, M.P., 2007. American College of Sports Medicine position stand. The female athlete triad. *Medicine and Science in Sports and Exercise*, 39 (10), 1867-1882.

339. Negishi-Koga, T. and Takayanagi, H., 2009. Ca2+-NFATc1 signaling is an essential axis of osteoclast differentiation. *Immunological Reviews*, 231 (1), 241-256.

340. Nelson, R.J., Marinovic, A.C., Moffatt, C.A., Kriegsfeld, L.J. and Kim, S., 1997. The effects of photoperiod and food intake on reproductive development in male deer mice (Peromyscus maniculatus). *Physiology and behavior*, 62 (5), 945-950.

341. Nielson, C.M., Srikanth, P. and Orwoll, E.S., 2012. Obesity and fracture in men and women: an epidemiologic perspective. *Journal of Bone and Mineral Research*, 27 (1), 1-10.

342. Nieves, J.W., Melsop, K., Curtis, M., Kelsey, J.L., Bachrach, L.K., Greendale, G., Sowers, M.F. and Sainani, K.L., 2010. Nutritional factors that influence change in bone density and stress fracture risk among young female cross-country runners. *American Academy of Physical Medicine and Rehabilitation*, 2 (8), 740-750.

343. Nieves, J.W., Ruffing, J.A., Zion, M., Tendy, S., Yavorek, T., Lindsay, R. and Cosman, F., 2016. Eating disorders, menstrual dysfunction, weight change and DMPA use predict bone density change in college-aged women. *Bone*, 84, 113-119.

344. Nikander, R., Sievänen, H., Heinonen, A., Daly, R.M., Uusi-Rasi, K. and Kannus, P., 2010. Targeted exercise against osteoporosis: a systematic review and meta-analysis for optimising bone strength throughout life. *BMC medicine*, 8 (1), 47.

345. Nishiyama, S., Tomoeda, S., Ohta, T., Higuchi, A. and Matsuda, I., 1988. Differences in basal and postexercise osteocalcin levels in athletic and nonathletic humans. *Calcified Tissue Int*ernational, 43 (3), 150 –154.

346. Nyman, J.S., Even, J.L., Jo, C.H., Herbert, E.G., Murry, M.R., Cockrell, G.E., Wahl, E.C., Bunn, R.C., Lumpkin, C.K., Fowlkes, J.L. and Thrailkill, K.M., 2011 Increasing duration of type 1 diabetes perturbs the strength-structure relationship and increases brittleness of bone. *Bone*, 48 (4), 733-740.

347. Oddie, G.W., Schenk, G., Angel, N.Z., Walsh, N., Guddat, L.W., de Jersey, J., Cassady, A.I., Hamilton, S.E. and Hume, D.A., 2000. Structure, function, and regulation of tartrate-resistant acid phosphatase. *Bone*, 27 (5), 575-584.

348. Olmedillas, H., González-Agüero, A., Moreno, L.A., Casajus, J.A. and Vicente-Rodríguez, G., 2012. Cycling and bone health: a systematic review. *BMC Medicine*, 10 (1), 169.

349. Onur, S., Haas, V., Bosy-Westphal, A., Hauer, M., Paul, T., Nutzinger, D., Klein, H., Müller, M.J., 2005. L-tri-iodothyronine is a major determinant of resting energy expenditure in underweight patients with anorexia nervosa and during weight gain. *European Journal of Endocrinology*, 152 (2), 179–184.

350. Otis, C.L., Drinkwater, B., Johnson, M., Loucks, A. and Wilmore, J., 1997. American College of Sports Medicine position stand. The Female Athlete Triad. *Medicine and Science in Sports and Exercise*, 29 (5), pp.i-ix.

351. Oury, F., Sumara, G., Sumara, O., Ferron, M., Chang, H., Smith, C.E., Hermo, L., Suarez, S., Roth, B.L., Ducy, P. and Karsenty, G., 2011. Endocrine regulation of male fertility by the skeleton. Cell, 144 (5), 796-809.

352. Pacheco-Pantoja, E.L., Ranganath, L.R., Gallagher, J.A., Wilson, P.J. and Fraser, W.D., 2011. Receptors and effects of gut hormones in three osteoblastic cell lines. *BMC physiology*, 11 (1), 1.

353. Petersen, S.R., Anderson, G.S., Tipton, M.J., Docherty, D., Graham, T.E., Sharkey, B.J. and Taylor, N.A., 2016. Towards best practice in physical and physiological employment standards. *Applied Physiology, Nutrition, and Metabolism*, 41 (6), S47-S62.

354. Pikkarainen, E., Lehtonen-Veromaa, M., Möttönen, T., Kautiainen, H. and Viikari, J., 2008. Estrogen–progestin contraceptive use during adolescence prevents bone mass acquisition: a 4-year follow-up study. *Contraception*, 78 (3), 226-231.

355. Polatti, F., Perotti, F., Filippa, N., Gallina, D. and Nappi, R.E., 1995. Bone mass and long-term monophasic oral contraceptive treatment in young women. Contraception, 51 (4), 221-224.

356. Pontzer, H., 2015. Constrained Total Energy Expenditure and the Evolutionary Biology of Energy Balance. *Exercise and Sport Sciences Reviews*, 43 (3), 110-116.

357. Poole, K.E. and Reeve, J., 2005. Parathyroid hormone—a bone anabolic and catabolic agent. *Current opinion in pharmacology*, 5 (6), 612-617.

358. Pritchard, J.E., Nowson, C.A. and Wark, J.D., 1996. Bone loss accompanying diet-induced or exercise-induced weight loss: a randomised controlled study. *International journal of obesity and related metabolic disorders*, 20 (6), 513–520.

359. Proff, P. and Römer, P., 2009. The molecular mechanism behind bone remodelling: a review. *Clinical Oral Investigations*, 13 (4), 355-362.

360. Prouteau, S., Benhamou, L. and Courteix, D., 2006. Relationships between serum leptin and bone markers during stable weight, weight reduction and weight regain in male and female judoists. *European Journal of Endocrinology*, 154 (3), 389-395.

361. Pun, K.K., Lau, P. and Ho, P.W.M., 1989. The characterization, regulation, and function of insulin receptors on osteoblast-like clonal osteosarcoma cell line. *Journal of Bone and Mineral Research*, 4 (6), 853-862.

362. Qvist, P., Christgau, S., Pedersen, B. J., Schlemmer, A., and Christiansen, C., 2002. Circadian variation in the serum concentration of C-terminal telopeptide of type I collagen (serum CTx): effects of gender, age, menopausal status, posture, daylight, serum cortisol, and fasting. *Bone*, 31 (1), 57-61.

363. Ralston, S.H. and Uitterlinden, A.G., 2010. Genetics of osteoporosis. *Endocrinology Reviews*, 31 (5), 629-6662.

364. Rauh, M.J., Nichols, J.F. and Barrack, M.T., 2010. Relationships among injury and disordered eating, menstrual dysfunction, and low bone mineral density in high school athletes: a prospective study. *Journal of Athletic Training*, 45 (3), 243-252.

365. Recker, R.R., Davies, K.M., Hinders, S.M., Heaney, R.P., Stegman M.R. and Kimmel D.B., 1992. Bone gain in young adult women. *Journal of the American Medical Association*, 268 (17), 2403–2408.

366. Rector, R.S., Loethen, J., Ruebel, M., Thomas, T.R. and Hinton, P.S., 2009. Serum markers of bone turnover are increased by modest weight loss with or without weight-bearing exercise in overweight premenopausal women. *Applied Physiology, Nutrition, and Metabolism*, 34 (5), 933-941.

367. Rector, R.S., Rogers, R., Ruebel, M. and Hinton, P.S., 2008. Participation in road cycling vs running is associated with lower bone mineral density in men. *Metabolism*, 57 (2), 226–232.

368. Redman, L.M., Rood, J., Anton, S.D., Champagne, C., Smith, S.R. and Ravussin, E., 2008. Calorie restriction and bone health in young, overweight individuals. *Archives of Internal Medicine*, 168 (17), 1859-1866.

369. Reed SD, Scholes D, LaCroix AZ, Ichikawa LE, Barlow WE and Ott SM., 2003. Longitudinal changes in bone density in relation to oral contraceptive use. *Contraception*, 68 (3), 177-82.

370. Reed, J.L., De Souza, M.J. and Williams, N.I., 2013. Changes in energy availability across the season in Division I female soccer players. *Journal of Sports Science*, 31 (3), 314-324.

371. Rickenlund, A., Thorén, M., Carlström, K., Von Schoultz, B.O. and Hirschberg, A.L., 2004. Diurnal profiles of testosterone and pituitary hormones suggest different mechanisms for menstrual disturbances in endurance athletes. *The Journal of Clinical Endocrinology & Metabolism*, 89 (2), 702-707.

372. Riedt, C.S., Schlussel, Y., von Thun, N., Ambia-Sobhan, H., Stahl, T., Field, M.P., Sherrell, R.M. and Shapses, S.A., 2007. Premenopausal overweight women do not lose bone during moderate weight loss with adequate or higher calcium intake. *The American journal of clinical nutrition*, 85 (4), 972-980.

373. Riggs, B. L., Khosla, S., and Melton, L.J., 1998. A unitary model for involutional osteoporosis: estrogen deficiency causes both type I and type II osteoporosis in postmenopausal women and contributes to bone loss in aging men. *Journal of Bone and Mineral Research*, 13 (5), 763-773.

374. Rissanen, J.P., Suominen, M.I., Peng, Z. and Halleen, J.M., 2008. Secreted tartrate-resistant acid phosphatase 5b is a Marker of osteoclast number in human osteoclast cultures and the rat ovariectomy model. *Calcified Tissue International*, 82 (2), 108-115.

375. Rizzoli, R., 2008. Nutrition: its role in bone health. *Best Practice in Research in Clinical Endocrinology and Metabolism*, 22 (5), 813-829.

376. Rizzoli, R., 2014. Nutritional aspects of bone health. *Best Practice in Research in Clinical Endocrinology and Metabolism*, 28 (6), 795-808.

377. Rizzoli, R., Bianchi, M.L., Garabédian, M., McKay, H.A. and Moreno, L.A., 2010. Maximizing bone mineral mass gain during growth for the prevention of fractures in the adolescents and the elderly. *Bone*, 46 (2), 294-305.

378. Robbeson, J.G., Havemann-Nel, L. and Wright, H.H., 2013. The female athlete triad in student track and field athletes. *South African Journal of Clinical Nutrition*, 26 (2), 19-24.

379. Roberts, D., Gebhardt, D.L., Gaskill, S.E., Roy, T.C. and Sharp, M.A., 2016. Current considerations related to physiological differences between the sexes and physical employment standards. *Applied Physiology Nutrition and Metabolism*, 41 (6 Suppl 2), S108-S120.

380. Robling, A.G., Castillo, A.B., Turner, C.H., 2006. Biomechanical and molecular regulation of bone remodeling. *Annual Review of Biomedical Engineering*, 8, 455-498.

381. Robling, A.G., Niziolek, P.J., Baldridge, L.A., Condon, K.W., Allen, M.R., Alam, I., Mantila, S.M., Gluhak-Heinrich, J., Bellido, T.M., Harris, S.E. and Turner, C.H., 2008. Mechanical stimulation of bone in vivo reduces osteocyte expression of Sost/sclerostin. *Journal of Biological Chemistry*, 283 (9), 5866-5875.

382. Rodriguez-Sanchez, N., and Galloway, S.D., 2015. Errors in dual energy X-ray absorptiometry estimation of body composition induced by hypohydration. *International journal of sport nutrition and exercise metabo*lism, 25 (1), 60-68.

383. Ronsen, O., Lea, T., Bahr, R. and Pedersen, B.K., 2002. Enhanced plasma IL-6 and IL-1ra responses to repeated vs. single bouts of prolonged cycling in elite athletes. *Journal of Applied Physiology*, 92 (6), 2547–2553.

384. Ronsen, O., Pedersen, B.K., Oritsland, T.R., Bahr, R., Kjeldsen-Kragh, J., 2001 Leukocyte counts and lymphocyte responsiveness associated with repeated bouts of strenuous endurance exercise. *Journal of Applied Physiology*, 91 (1), 425–434.

385. Roza, A.M., and Shizgal, H.M., 1984. The Harris Benedict equation reevaluated: resting energy requirements and the body cell mass. *The American Journal of Clinical Nutrition*, 40 (1), 168-182.

386. Ruohola, J.P., Laakksi, I., Ylikomi, T., Haatja, R., Mattila, V.M., Sahi, T., Tuohimaa, P. and Pihlajamaki, H., 2006. Association between serum 25(OH)D concentrations and bone stress fractures in Finnish young men. *Journal of Bone and Mineral Research*. 21 (9), 1483-1488.

387. Russell, M., Stark, J., Nayak, S., Miller, K.K., Herzog, D.B., Klibanski, A. and Misra, M., 2009. Peptide YY in adolescent athletes with amenorrhea, eumenorrheic athletes and non-athletic controls. *Bone*, 45 (1), 104-109.

388. Ryan, A.S., Nicklas, B.J. and Dennis, K.E., 1998. Aerobic exercise maintains regional bone mineral density during weight loss in postmenopausal women. *Journal of Applied Physiology*, 84, 1305–1310.

389. Sadler, C., Smith, H., Hammond, J., Bayly, R., Borland, S., Panay, N., Crook, D. and Inskip, H., 2010. Lifestyle factors, hormonal contraception, and premenstrual symptoms: the United Kingdom Southampton Women's Survey. *Journal of Women's Health*, 19 (3), 391-396.

390. Saris, W.H., 2001. Very-Low-Calorie Diets and Sustained Weight Loss. *Obesity Research*, 9 (S11), 295S-301S.

391. Schaal, K.., Tiollier, E., Le Meur, Y., Casazza, G. and Hausswirth, C., 2016. Elite synchronized swimmers display decreased energy availability during intensified training. *Scandinavian Journal of Medicine & Science in Sports.* doi: 10.1111/sms.12716.

392. Schafer, A.L., 2016. Decline in Bone Mass During Weight Loss: A Cause for Concern? *Journal of Bone and Mineral Research*, 31 (1), 36-39.

393. Scheid, J.L., Toombs, R.J., Ducher, G., Gibbs, J.C., Williams, N.I. and De Souza, M.J., 2011. Estrogen and peptide YY are associated with bone mineral density in premenopausal exercising women. *Bone*, 49 (2), 194-201.

394. Schipilow, J.D., Macdonald, H.M., Liphardt, A.M., Kan, M. and Boyd, S.K., 2013. Bone micro-architecture, estimated bone strength, and the muscle-bone interaction in elite athletes: an HR-pQCT study. *Bone*, 56 (2), 281-289.

395. Schlemmer, A., Hassager, C.H., Jensen, S.B., Christiansen, C.L., 1992. Marked diurnal variation in urinary excretion of pyridinium cross-links in premenopausal women. *The Journal of Clinical Endocrinology & Metabolism*, 74 (3), 476-480.

396. Scholes, D., Hubbard, R.A., Ichikawa, L.E., LaCroix, A.Z., Spangler, L., Beasley, J.M., Reed, S. and Ott, S.M., 2011. Oral contraceptive use and bone density change in adolescent and young adult women: a prospective study of age, hormone dose, and discontinuation. *The Journal of Clinical of Endocrinology and Metabolism*, 96 (9), E1380-E1387.

397. Scholes, D., Ichikawa, L., LaCroix, A.Z., Spangler, L., Beasley, J.M., Reed, S. and Ott, S.M., 2010. Oral contraceptive use and bone density in adolescent and young adult women. *Contraception*, 81 (1), 35-40.

398. Schreml, S., Szeimies, R.M., Prantl, L., Landthaler, M. and Babilas, P., 2010. Wound healing in the 21st century. *Journal of the American Academy of Dermatology*, 63 (5), 866-881.

399. Scibora, L.M., 2014. Skeletal effects of bariatric surgery: examining bone loss, potential mechanisms and clinical relevance. *Diabetes Obesity and Metabolism*, 16 (12), 1204-1213.

400. Scibora, L.M., Ikramuddin, S., Buchwald, H. and Petit, M.A., 2012. Examining the link between bariatric surgery, bone loss, and osteoporosis: a review of bone density studies. *Obesity surgery*, 22 (4), 654-667.

401. Scofield, K.L. and Hecht, S., 2012. Bone health in endurance athletes: runners, cyclists, and swimmers. *Current Sports Medicine Reports*, 11 (6), 328-334.

402. Scott, J.P., Sale, C., Greeves, J.P., Casey, A., Dutton, J. and Fraser, W.D., 2010. The effect of training status on the metabolic response of bone to an acute bout of exhaustive treadmill running. *The Journal of Clinical Endocrinology and Metabolism*, 95 (8), 3918-3925.

403. Scott, J.P., Sale, C., Greeves, J.P., Casey, A., Dutton, J. and Fraser, W.D., 2012. Effect of fasting versus feeding on the bone metabolic response to running. *Bone*, 51 (6), 990-999.

404. Scott, J.P., Sale, C., Greeves, J.P., Casey, A., Dutton, J. and Fraser, W.D., 2013. Effect of recovery duration between two bouts of running on bone metabolism. *Medicine and Science in Sports and Exercice*, 45 (3), 429-438.

405. Seibel, M.J., 2005. Biochemical markers of bone turnover part I: biochemistry and variability. The Clinical biochemist. *Reviews/Australian Association of Clinical Biochemists*, 26 (4), 97.

406. Shah, K., Armamento-Villareal, R., Parimi, N., Chode, S., Sinacore, D.R., Hilton, T.N., Napoli, N., Qualls, C. and Villareal, D.T., 2011. Exercise training in obese older adults prevents increase in bone turnover and attenuates decrease in hip bone mineral density induced by weight loss despite decline in bone-active hormones. *Journal of Bone and Mineral Research*, 26 (12), 2851-2859.

407. Shapses, S.A. and Sukumar, D., 2012. Bone metabolism in obesity and weight loss. *Annual Reviews of Nutrition*, 32, 287-309.

408. Shepherd, J.A., Fan, B., Lu, Y., Lewiecki, E.M., Miller, P. and Genant, H.K., 2006 Comparison of BMD precision for Prodigy and Delphi spine and femur scans. *Osteoporosis International*. 17 (9), 1303-1308.

409. Shiga, K., Hara, H., Okano, G., Ito, M., Minami, A. and Tomita, F., 2003. Ingestion of difructose anhydride III and voluntary running exercise independently increase femoral and tibial bone mineral density and bone strength with increasing calcium absorption in rats. *The Journal of nutrition*, 133 (12), 4207-4211.

410. Sibonga, J.D., Spector, E.R., Johnston, S.L. and Tarver, W.J., 2015. Evaluating bone loss in ISS astronauts. *Aerospace medicine and human performance*, 86 (12), A38-A44.

411. Skov, A.R., Haulrik, N., Toubro, S., Molgaard, C. and Astrup, A., 2002. Effect of protein intake on bone mineralization during weight loss: a 6-month trial. *Obesity Research*, 10 (6), 432-438.

412. Slater, J., McLay-Cooke, R., Brown, R. and Black, K., 2016. Female Recreational Exercisers at Risk of Low Energy Availability. *International journal of sport nutrition and exercise metabolism*, 24, 1-18.

413. Smith, E.P., Boyd, J., Frank, G.R., Takahashi, H., Cohen, R.M., Specker, B., Williams, T.C., Lubahn, D.B. and Korach, K.S., 1994. Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. New England Journal of Medicine, 331 (16), 1056-1061.

414. Snyder, R.A., Koester, M.C. and Dunn, W.R., 2006. Epidemiology of stress fractures. *Clinics in sports medicine*, 25 (1), 37-52.

415. Soltani, S., Hunter, G.R., Kazemi, A. and Shab-Bidar, S., 2016. The effects of weight loss approaches on bone mineral density in adults: a systematic review and meta-analysis of randomized controlled trials. Osteoporosis International, 27 (9), 2655-2671.

416. Sowers, M.R., Clark, M.K., Hollis, B., Wallace, R.B., Jannausch, M., 1992. Radial bone mineral density in pre- and perimenopausal women: a prospective study of rates and risk factors for loss. *Journal of Bone and Mineral Research*, 7 (6), 647-657.

417. Spatz, J.M., Ellman, R., Cloutier, A.M., Louis, L., van Vliet, M., Suva, L.J., Dwyer, D., Stolina, M., Ke, H.Z. and Bouxsein, M.L,, 2013. Sclerostin antibody inhibits skeletal deterioration due to reduced mechanical loading. *Journal of Bone and Mineral Research*, 28 (4), 865-874.

418. Spector, E.R., Smith, S.M. and Sibonga, J.D., 2009. Skeletal effects of long-duration head-down bed rest. *Aviation, space, and environmental medicine*, 80 (Supplement 1), A23-A28.

419. St-Arnaud, R., 2008. The direct role of vitamin D on bone homeostasis. *Archives of Biochemical Biophysics*, 473 (2), 225-230.

420. Stolzenberg, N., Belavý, D.L., Beller, G., Armbrecht, G., Semler, J. and Felsenberg, D., 2003. Bone strength and density via pQCT in post-menopausal osteopenic women after 9 months resistive exercise with whole body vibration or proprioceptive exercise. *Journal of Musculoskeletal and Neuronal Interactions*, 13 (1), 66-76.

421. Stricker, R., Eberhart, R., Chevailler, M.C., Quinn, F.A., Bischof, P. and Stricker. R., 2006. Establishment of detailed reference values for luteinizing hormone, follicle stimulating hormone, estradiol, and progesterone during different phases of the menstrual cycle on the Abbott ARCHITECT® analyser. *Clinical Chemical Laboratory Medicine*, 44 (7), 883-887.

422. Stubbs, R.J., Hughes, D.A., Johnstone, A.M., Horgan, G.W., King, N. and Blundell, J.E., 2004. A decrease in physical activity affects appetite, energy, and nutrient balance in lean men feeding ad libitum. *American Journal of Clinical Nutrition*, 79 (1), 62-69.

423. Sundgot-Borgen, J. and Torstveit, M.K., 2004. Prevalence of eating disorders in elite athletes is higher than in the general population. *Clinical Journal of Sport Medicine*, 14 (1), 25-32.

424. Sundgot- Borgen, J. and Torstveit, M.K., 2010. Aspects of disordered eating continuum in elite high- intensity sports. Scandinavian Journal of Medicine & Science in Sports, 20 (s2), 112-121.

425. Svendsen, O.L., Hassager, C. and Christiansen, C., 1993. Effect of an energy-restrictive diet, with or without exercise, on lean tissue mass, resting metabolic rate, cardiovascular risk factors, and bone in overweight postmenopausal women. *American Journal of Medicine*, 95 (2), 131-140. Sweeney, G., 2010. Cardiovascular effects of leptin. Nature Reviews Cardiology, 7 (1), 22-29.

426. Swift SN, Baek K, Swift JM, Bloomfield SA., 2012. Restriction of dietary energy intake has a greater impact on bone integrity than does restriction of calcium in exercising female rats. *Journal of Nutrition*, 142 (6), 1038-1045.

427. Szulc, P. and Delmas, P.D., 2008. Biochemical markers of bone turnover: potential use in the investigation and management of postmenopausal osteoporosis. *Osteoporosis International*, 19 (12), 1683-1704.

428. Taaffe, D.R., Robinson, T.L., Snow, C.M. and Marcus, R., 1997. High-Impact Exercise Promotes Bone Gain in Well-Trained Female Athletes. *Journal of Bone and Mineral Research*, 12 (2), 255-260.

429. Takeda, S., Elefteriou, F., Levasseur, R., Liu, X., Zhao, L., Parker, K.L., Armstrong, D., Ducy, P. and Karsenty, G., 2002. Leptin regulates bone formation via the sympathetic nervous system. *Cell*, 111 (3), 305-317.

430. Talbott, S.M., Cifuentes, M., Dunn, M.G. and Shapses, S.A., 2001. Energy restriction reduces bone density and biomechanical properties in aged female rats. *The Journal of nutrition*, 131 (9), 2382-2387.

431. Talbott, S.M., Rothkopf, M.M. and Shapses, S.A., 1998. Dietary restriction of energy and calcium alters bone turnover and density in younger and older female rats. *The Journal of nutrition*, 128 (3), 640-645.
432. Tang, B.M., Eslick, G.D., Nowson, C., Smith, C. and Bensoussan, A., 2007. Use of calcium or calcium in combination with vitamin D supplementation to prevent fractures and bone loss in people aged 50 years and older: a meta-analysis. *The Lancet*, 370 (9588), 657-666.
433. Teff, K.L., 2010. Cephalic phase pancreatic polypeptide responses to liquid and solid stimuli in humans. *Physiological Behavior*, 99 (3), 317-323.
434. Teitelbaum, S.L., 2007. Osteoclasts: what do they do and how do they do it? *American Journal of Pathology*, 170 (2), 427-435.
435. Tenforde, A.S. and Fredericson, M., 2011. Influence of sports participation on bone health in the young athlete: a review of the literature. *Physical Medicine and Rehabilitation*, 3 (9), 861–867.
436. Tenforde, A.S., Barrack, M.T., Nattiv, A. and Fredericson, M., 2016. Parallels with the female athlete triad in male athletes. *Sports Medicine*, 46 (2), 171-182.
437. Tenforde, A.S., Sainani, K.L., Sayres, L.C., Cutti, P. and Fredericson, M., 2015. Identifying sex-specific risk factors for low bone mineral density in adolescent runners. *American Journal of Sports Medicine*, 43 (6), 1494–1504.
438. Tharion, W.J., Lieberman, H.R., Montain, S.J., Young, A.J., Baker-Fulco, C.J., Delany, J.P. and Hoyt, R.W., 2005. Energy requirements of military personnel. *Appetite*, 44 (1), 47–65.
439. Thein-Nissenbaum, J.M., Carr, K.E., Hetzel, S., Dennison, E., 2014. Disordered eating, menstrual irregularity, and musculoskeletal injury in high school athletes: a comparison of oral contraceptive pill users and nonusers. *Sports Health*, 6 (4), 313-320.
440. Thiemann, P., Legenbauer, T., Vocks, S., Platen, P., Auyeung, B. and Herpertz, S., 2015. Eating disorders and their putative risk factors among female German professional athletes. *European Eating Disorders Review*, 23 (4), 269-276.
441. Thong, F.S., McLean, C. and Graham, T.E., 2000. Plasma leptin in female athletes: relationship with body fat, reproductive, nutritional, and endocrine factors. *Journal of Applied Physiology*, 88 (6), 2037-2044.
442. Thorpe, M.P., Jacobson, E.H., Layman, D.K., He, X., Kris-Etherton, P.M. and Evans, E.M., 2008. A diet high in protein, dairy, and calcium attenuates bone loss over twelve months of weight loss and maintenance relative to a conventional high-carbohydrate diet in adults. *Journal of Nutrition*, 138 (6), 1096-1100.
443. Tomasik, P.J., Sztefko, K. and Starzyk, J., 2004. Cholecystokinin, glucose dependent insulinotropic peptide and glucagon-like peptide 1 secretion in children with anorexia nervosa and simple obesity. *Journal of Pediatric Endocrinology and Metabolism*, 17 (12), 1623-1632.
444. Torstveit, M.K. and Sundgot-Borgen, J., 2005. The female athlete triad: are elite athletes at increased risk?. *Medicine & Science in Sports and Exercise*, 37 (2), 184-193.
445. Turner, A.S., 2001b. Animal models of osteoporosis—necessity and limitations. European Cell Material, 1 (66-81), 13-29.

446. Turner, C.H. and Robling, A.G., 2003. Designing exercise regimens to increase bone strength. *Exercise and Sport Science Reviews*, 31 (1), 45-50.

447. Turner, C.H., 2002. Biomechanics of bone: determinants of skeletal fragility and bone quality. *Osteoporosis International*, 13 (2), 97-104.

448. Turner, R.T., 2001a. Skeletal adaptation to external loads optimizes mechanical properties: fact or fiction. *Current Opinions in Orthopedics*, 12 (5), 384-388.

449. Túry, F., Güleç, H. and Kohls, E., 2010. Assessment methods for eating disorders and body image disorders. *Journal of Psychosomatic Research*, 69 (6), 601-611.

450. Uitterlinden, A.G., Zillikens, C.M. and Rivadeneira, F., 2013 Genetic Determinants of Osteoporosis. In: Osteoporosis, 4th Edition, Waltham, Academic Press.

451. Ulrich, U., Miller, P.B., Eyre, D.R., Chesnut III, C.H., Schlebusch, H. and Soules, M.R., 2003. Bone remodeling and bone mineral density during pregnancy. *Archives of gynecology and obstetrics*, 268 (4), 309-316

452. Upadhyay, J., Farr, O.M. and Mantzoros, C.S., 2015. The role of leptin in regulating bone metabolism. *Metabolism*, 64 (1), 105-113.

453. Vainionpaa, A., Korpelainen, R., Sievanen, H., Vihriala, E., Leppaluoto, J. and Jamsa, T., 2007. Effect of impact exercise and its intensity on bone geometry at weight-bearing tibia and femur. *Bone*, 40 (3), 604-611.

454. van den Hooven, E.H., Ambrosini, G.L., Huang, R.C., Mountain, J., Straker, L., Walsh, J.P., Zhu, K. and Oddy WH., 2015 Identification of a dietary pattern prospectively associated with bone mass in Australian young adults. *American Journal of Clinical Nutrition*. 102 (5), 1035-1043.

455. Vanderschueren, D., Laurent, M.R., Claessens, F., Gielen, E., Lagerquist, M.K., Vandenput, L., Börjesson, A.E. and Ohlsson, C., 2014. Sex steroid actions in male bone. *Endocrinology Reviews*, 35 (6), 906-960.

456. Vanderschuerren, D., Vandenput, L., Boonen, S., Lindberg, M.K., Bouillon, R., Ohlsson, C., 2003. Androgens and Bone. *Endocrinology Reviews*, 25 (3), 389-425.

457. Varley, I., Hughes, D.C., Greeves, J.P., Stellingwerff, T., Ranson, C., Fraser, W.D. and Sale, C., 2015. RANK/RANKL/OPG pathway: Genetic associations with stress fracture period prevalence in elite athletes. *Bone*, 71, 131-136.

458. Vasikaran, S., Eastell, R., Bruyère, O., Foldes, A.J., Garnero, P., Griesmacher, A., McClung, M., Morris, H.A., Silverman, S., Trenti, T., Wahl, D.A., Cooper, C., Kanis, J.A. and IOF-IFCC Bone Marker Standards Working Group, 2011.Markers of bone turnover for the prediction of fracture risk and monitoring of osteoporosis treatment: a need for international reference standards. *Osteoporosis International*, 22 (2), 391–420.

459. Veitch, S.W., Findlay, S.C., Hamer, A.J., Blumsohn, A., Eastell, R. and Ingle, B.M., 2006. Changes in bone mass and bone turnover following tibial shaft fracture. *Osteoporosis International*, 17 (3), 364-372.

460. Villareal, D.T., Fontana, L., Das, S.K., Redman, L., Smith, S.R., Saltzman, E., Bales, C., Rochon, J., Pieper, C., Huang, M. and Lewis, M., 2016. Effect of Two-Year Caloric Restriction

on Bone Metabolism and Bone Mineral Density in Non-obese Younger Adults: a Randomized Clinical Trial. *Journal of Bone and Mineral Research*, 31 (1), 40-51.

461. Villareal, D.T., Fontana, L., Weiss, E.P., Racette, S.B., Steger-May, K., Schechtman, K.B., Klein, S. and Holloszy, J.O., 2006. Bone mineral density response to caloric restriction-induced weight loss or exercise-induced weight loss: a randomized controlled trial. *Archives of Internal Medicine*, 166 (22), 2502-2510.
462. Villareal, D.T., Shah, K., Banks, M.R., Sinacore, D.R. and Klein, S., 2008. Effect of weight loss and exercise therapy on bone metabolism and mass in obese older adults: a one-year randomized controlled trial. *The Journal of Clinical Endocrinology and Metabolism*, 93 (6), 2181-2187.
463. Vincent, K.R. and Braith, R.W., 2002. Resistance exercise and bone turnover in elderly men and women. *Medicine and Science in Sports and Exercice*, 34 (1), 17-23.
464. Viner, R.T., Harris, M., Berning, J.R. and Meyer, N.L., 2015. Energy Availability and Dietary Patterns of Adult Male and Female Competitive Cyclists With Lower Than Expected Bone Mineral Density. *International Journal of Sport Nutrition and Exercise Metabolism*, 25 (6), 594-602.
465. Viru, A., Karelson, K. and Smirnova, T., 1992. Stability and variability in hormonal responses to prolonged exercise. *International journal of sports medicine*, 13 (3), 230-235.
466. Vogt, S., Heinrich, L., Schumacher, Y.O., Grosshauser, M., Blum, A., König, D., Berg, A., Schmid, A., 2005. Energy intake and energy expenditure of elite cyclists during preseason training. *International Journal of Sports Medicine*, 26 (8), 701-716.
467. Wacker, M. and Holick, M.F., 2013. Vitamin D- effects on skeletal and extraskeletal health and the need for supplementation. *Nutrients*, 5 (1), 111-148.
468. Wade GN and Jones, J.E., 2004.Neuroendocrinology of nutritional infertility. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*, 287 (6), R1277-R1296.
469. Wade, G.N., Schneider, J.E. and Li, H.Y., 1996. Control of fertility by metabolic cues. *American Journal of Physiology*, 270 (1 Pt 1), E1–E19.
470. Waldron-Lynch, F., Murray, B.F., Brady, J.J., McKenna, M.J., McGoldrick, A., Warrington, G., O'Loughlin, G. and Barragry, J.M., 2010. High bone turnover in Irish professional jockeys. *Osteoporosis International*, 21 (3), 521-525. Walsh, J.S. and Henriksen, D.B., 2010. Feeding and bone. *Archives of Biochemical Biophysics*, 503 (1), 11-19.
471. Warden, S.J., Burr, D.B. and Brukner, P.D., 2006. Stress fractures: pathophysiology, epidemiology, and risk factors. *Current Osteoporosis Reports*, 4 (3), 103-109.
472. Warden, S.J., Davis, I.S. and Fredericson, M., 2014. Management and prevention of bone stress injuries in long-distance runners. *The Journal of Orthopaedic and Sports Physical Therapy*, 44 (10), 749-765.
473. Warden, S.J., Hurst, J.A., Sanders, M.S., Turner, C.H., Burr, D.B. and Li, J., 2005. Bone adaptation to a mechanical loading program significantly increases skeletal fatigue resistance. *Journal of Bone and Mineral Research*, 20 (5), 809-816.

474. Wardle, J., Haase, A.M. and Steptoe, A., 2006. Body image and weight control in young adults: international comparisons in university students from 22 countries. *International Journal of Obesity (Lond)*, 30 (4), 644-651.

475. Warrington, G., Dolan, E., McGoldrick A, McEvoy, J., Macmanus, C., Griffin, M. and Lyons, D., 2009. Chronic weight control impacts on physiological function and bone health in elite jockeys. *Journal of Sports Science*, 27 (6), 543–550.

476. Waugh, E.J., Lam, M.A., Hawker, G.A., McGowan, J., Papaioannou, A., Cheung, A.M., Hodsman, A.B., Leslie, W.D., Siminoski, K. and Jamal, S.A., 2009. Risk factors for low bone mass in healthy 40–60 year old women: a systematic review of the literature. *Osteoporosis international*, 20 (1), 1-21.

477. Waung, J.A., Bassett, J.D. and Williams, G.R., 2012. Thyroid hormone metabolism in skeletal development and adult bone maintenance. *Trends in Endocrinology and Metabolism*, 23 (4), 155-162.

478. Weaver, C.M., Gordon, C.M., Janz, K.F., Kalkwarf, H.J., Lappe, J.M., Lewis, R., O'Karma, M., Wallace, T.C. and Zemel, B.S., 2016. The National Osteoporosis Foundation's position statement on peak bone mass development and lifestyle factors: a systematic review and implementation recommendations. *Osteoporosis International*, 27 (4), 1281-1386.

479. Weaver, C.M., Teegarden, D., Lyle, R.M., McCabe, G.P., McCabe, L.D., Proulx, W., Kern, M., Sedlock, D., Anderson, D.D., Hillberry, B.M., Peacock, M. and Johnston, C.C., 2001. Impact of exercise on bone health and contraindication of oral contraceptive use in young women. *Medicine and Science in Sports and Exercice*, 33 (6), 873-880.

480. Wee, N.K., Kulkarni, R.N., Horsnell, H. and Baldock, P.A., 2016. The brain in bone and fuel metabolism. *Bone*, 82, 56-63.

481. Wehrli, F.W., Song, H.K., Saha, P.K. and Wright, A.C., 2006. Quantitative MRI for the assessment of bone structure and function. NMR Biomedicine, 19 (7), 731–764.

482. Welsh, L., Rutherford, O.M., James, I., Crowley, C., Comer, M. and Wolman, R., 1997. The acute effects of exercise on bone turnover. *International journal of sports medicine*, 18 (4), 247-251.

483. Welt, C.K., Chan, J.L., Bullen, J., Murphy, R., Smith, P., DePaoli, A.M., Karalis, A. and Mantzoros, C.S., 2004. Recombinant human leptin in women with hypothalamic amenorrhea. *New England Journal of Medicine*, 351 (10), 987-997.

484. Wentz, L., Liu, P.Y., Haymes, E. and Ilich, J.Z., 2011. Females have a greater incidence of stress fractures than males in both military and athletic populations: a systemic review. *Military Medicine*, 176 (4), 420-430.

485. Wesner, M. L., 2012. Nutrient effects on stress reaction to bone. Canadian Family Physician, 58 (11), 1226–1230.

486. Westerterp, K.R., 2004. Diet induced thermogenesis. *Nutrition and metabolism*, 1 (1), 5.

487. Wheater, G., Elshahaly, M., Tuck, S. P., Datta, H. K. and van Laar, J. M., 2013. The clinical utility of bone marker measurements in osteoporosis. *Journal of Translational Medicine*, 11, 201-215.

488. Wheeler, G.D., Singh, M., Pierce, W.D., Epling, W.F. and Cumming, D.C., 1991. Endurance training decreases serum testosterone levels in men without change in luteinizing hormone pulsatile release. *The Journal of Clinical Endocrinology and Metabolism*, 72 (2), 422-425.

489. Wiegratz, I., Kutschera, E., Lee, J.H., Moore, C., Mellinger, U., Winkler, U.H. and Kuhl, H., 2003. Effect of four different oral contraceptives on various sex hormones and serum-binding globulins. *Contraception*, 67 (1), 25-32.

490. Williams, N.I., Leidy, H.J., Hill, B.R., Lieberman, J.L., Legro, R.S. and De Souza, M.J., 2015. Magnitude of daily energy deficit predicts frequency but not severity of menstrual disturbances associated with exercise and caloric restriction. *American Journal of Physiology Endocrinology and Metabolism*, 308 (1), E29-E39.

491. Williams, R.L., Wood, L.G., Collins, C.E. and Callister, R., 2015. Effectiveness of weight loss interventions–is there a difference between men and women: a systematic review. *Obesity Reviews*, 16 (2), 171-186.

492. Wilson, G., Drust, B., Morton, J.P. and Close, G.L., 2014. Weight-making strategies in professional jockeys: implications for physical and mental health and well-being. *Sports Medicine*, 44 (6), 785-796.

493. Wilson, G., Fraser, W.D., Sharma, A., Eubank, M., Drust, B., Morton, J.P. and Close, G.L., 2013. Markers of bone health, renal function, liver function, anthropometry and perception of mood: a comparison between Flat and National Hunt Jockeys. *International Journal of Sports Medicine*, 34 (5), 453-459.

494. Winfield, A.C., Moore, J., Bracker, M. and Johnson, C.W., 1997. Risk factors associated with stress reactions in female Marines. *Military medicine*, 162 (10), 698-702.

495. Winzenberg, T., Shaw, K., Fryer, J. and Jones, G., 2006. Effects of calcium supplementation on bone density in healthy children: meta-analysis of randomised controlled trials. *BMJ*, 333 (7572), 775.

496. Woitge, H.W., Friedmann, B., Suttner, S., Farahmand, I., Müller, M., Schmidt-Gayk, H., Baertsch, P., Ziegler, R. and Seibel, M.J., 1998. Changes in bone turnover induced by aerobic and anaerobic exercise in young males. *Journal of Bone and Mineral Research*, 13 (12), 1797-1804.

497. Wojcik, M.H., Meenaghan, E., Lawson, E.A., Misra, M., Klibanski, A. and Miller, K.K., 2010. Reduced amylin levels are associated with low bone mineral density in women with anorexia nervosa. *Bone*, 46(3), 796-800.

498. Wolff, J., 1986. The law of bone remodeling [translated from the 1892 original, Das Gesetz der Transformation der Knochen, by P. Maquet and R. Furlong]. Berlin: Springer Verlag.

499. Yakar, S. and Rosen, C.J., 2003. From mouse to man: redefining the role of insulin-like growth factor-I in the acquisition of bone mass. *Experimental Biology and Medicine*, 228 (3), 245-252.

500. Yamada, C., Yamada, Y., Tsukiyama, K., Yamada, K., Udagawa, N., Takahashi, N., Tanaka, K., Drucker, D.J., Seino, Y. and Inagaki, N., 2008. The murine glucagon-like peptide-1 receptor is essential for control of bone resorption. *Endocrinology*, 149 (2), 574-579.

501. Yeager KK, Agostini R, Nattiv A. and Drinkwater. B.L., 1993. The female athlete triad: disordered eating, amenorrhea, osteoporosis. *Medicine and Science in Sports and Exercise*, 25 (7), 775–777.
502. Young, N., Formica, C., Szmukler, G. and Seeman, E., 1994. Bone density at weight-bearing and nonweight-bearing sites in ballet dancers: the effects of exercise, hypogonadism, and body weight. *The Journal of Clinical Endocrinology and Metabolism*, 78 (2), 449-544.
503. Zanker CL and Swaine IL, 1998b. Relation between bone turnover, oestradiol, and energy balance in women distance runners. *British Journal of Sports Medicine*, 32 (2), 167-171.
504. Zanker CL and Swaine IL, 2000. Responses of bone turnover markers to repeated endurance running in humans under conditions of energy balance or energy restriction. *European Journal of Applied Physiology*, 83 (4-5), 434-440.
505. Zanker, C.L. and Swaine, I.L., 1998a. Bone turnover in amenorrhoeic and eumenorrhoeic women distance runners. *Scandinavian Journal of Medicine and Science in Sports*, 8 (1), 20-26.
506. Zborowski JV, Talbott EO and Cauley JA, 2001. Polycystic ovary syndrome, androgen excess, and the impact on bone. *Obstetrics and Gynecology Clinics of North America*, 28 (1), 135-151.
507. Zerwekh, J.E., Ruml, L.A., Gottschalk, F. and Pak, C.Y., 1998. The effects of twelve weeks of bed rest on bone histology, biochemical markers of bone turnover, and calcium homeostasis in eleven normal subjects. *Journal of Bone and Mineral Research*, 13 (10), 1594-1601.
508. Zibellini, J., Seimon, R.V., Lee, C.M., Gibson, A.A., Hsu, M.S., Shapses, S.A., Nguyen, T.V. and Sainsbury, A., 2015. Does Diet-Induced Weight Loss Lead to Bone Loss in Overweight or Obese Adults? A Systematic Review and Meta-Analysis of Clinical Trials. *Journal of Bone and Mineral Research*, 30 (12), 2168-2178.
509. Zipfel, S., Mack, I., Baur, L.A., Hebebrand, J., Touyz, S., Herzog, W., Abraham, S., Davies, P.S. and Russell, J., 2013. Impact of exercise on energy metabolism in anorexia nervosa. *Journal of Eating Disorders*, 1 (1), 37.
510. Zittermann, A., Schwarz, I., Scheld, K., Sudhop, T., Berthold, H.K., Von Bergmann, K., Van der Ven, H. and Stehle, P., 2000. Physiologic fluctuations of serum estradiol levels influence biochemical markers of bone resorption in young women. *The Journal of Clinical Endocrinology & Metabolism*, 85 (1), 95-101.
511. Zweig, M.H. and Campbell, G., 1993. Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. *Clinical Chemistry*, 39 (4), 561-577.

Chapter 11- Appendices

Appendix 1. Health Screen Questionnaire

Name.....

Please complete this brief questionnaire to confirm fitness to participate:

1. **At present,** do you have any health problem for which you are:

(a) on medication, prescribed or otherwise Yes ☐ No ☐

(b) attending your general practitioner Yes ☐ No ☐

(c) on a hospital waiting list Yes ☐ No ☐

2. **In the past two years,** have you had any illness which require you to:

(a) consult your GP Yes ☐ No ☐

(b) attend a hospital outpatient department Yes ☐ No ☐

(c) be admitted to hospital Yes ☐ No ☐

3. **Have you ever** had any of the following?

(a) Convulsions/epilepsy Yes ☐ No ☐

(b) Asthma Yes ☐ No ☐

(c) Eczema Yes ☐ No ☐

(d) Diabetes Yes ☐ No ☐

(e) A blood disorder Yes ☐ No ☐

(f) Head injury Yes ☐ No ☐

(g) Digestive problems Yes ☐ No ☐

(h) Heart problems Yes ☐ No ☐

(i) Problems with bones or joints Yes ☐ No ☐

(j) Disturbance of balance / coordination Yes ☐ No ☐

(k) Numbness in hands or feet Yes ☐ No ☐

(l) Disturbance of vision Yes ☐ No ☐

(m) Ear / hearing problems Yes ☐ No ☐

(n) Thyroid problems Yes ☐ No ☐

(o) Kidney or liver problems Yes ☐ No ☐

(p) Allergy to nuts, alcohol etc Yes ☐ No ☐

(q) Any problems affecting your nose e.g. recurrent nose bleeds Yes ☐ No ☐

(r) Any nasal fracture or deviated nasal septum Yes ☐ No ☐

4. **Has any,** otherwise healthy, member of your family under the age of 50 died suddenly during or soon after exercise? Yes ☐ No ☐

5. Are there any reasons why blood sampling may be difficult? Yes ☐ No ☐

6. Have you had a blood sample taken previously? Yes ☐ No ☐

7. Have you had a cold, flu or any flu like symptoms in the last Month? Yes ☐ No

Continued…

Women only

8. Are you pregnant, trying to become pregnant or breastfeeding?Yes ☐ No ☐

If YES to any question, please describe briefly if you wish (egg to confirm problem was/is short-lived, insignificant or well controlled.)

.....
.....
.....
.....

Appendix 2. Menstrual Cycle Details

(All information is fully confidential)

Please circle the answer where appropriate.

Number:

Age:

Date of birth:

1) Have you had regular periods in the last six months? YES NO

2) How long in days is your menstrual cycle, from day 1 of bleeding (period) to day 1 of the next period? _____DAYS

3) Is the above time the same between periods? YES NO

If the answer was NO, please state the irregularity:

_____

4) How many days does your menstrual (blood) flow last? _____DAYS

5) Do you get pain during your period? YES NO

If YES, please state the symptoms and the days during the cycle when you suffer:

_____

6) Do you avoid exercise during your period? YES NO

If YES, please state your reasons for avoiding exercise:

_____

7) Do you take any medication or hormones to regulate your menstrual cycle?
YES NO

If YES, please state what you take and how often?

_____

8) Do you take any other medication? YES NO
If YES, please state what you take and how often?

_____

9) Have you previously used any form of hormonal contraception (oral contraceptive, implant, injection, and coil)? YES NO

If YES, please state the type of contraception used and the date that you ceased using it?

_____

10) When did you have your last period (day 1)?

_____

Appendix 3. Oral Contraceptive Details

(All information is fully confidential)

Please circle the answer where appropriate.

Number:

Age:

Date of birth:

1) What brand or oral contraceptive do you take?_____

2) How long have you been using your current oral contraceptive?_____

3) What date did you begin taking your current pack of pills?_____

4) What time of day do you usually consume the pill?_____

5) Will you be continuing to take the oral contraceptive for the next 2 months?

YES NO

6) Do you take any other medication or hormones to regulate your cycle?

YES NO

If YES, please state what you take and how often?

_____

7) Do you take any other medication? YES NO

If YES, please state what you take and how often?

_____

Appendix 4. SCOFF questionnaire and weight history

Take a few minutes to answer these questions.

1) Do you ever make yourself sick because you feel uncomfortably full? _____

2) Do you worry you have lost control over how much you eat? ____

3) Have you recently lost more than 5 kg in a three month period? _____

4) Do you believe yourself to be fat when others say you are too thin? _____

5) Would you say that food dominates your life? _____

6) Your usual weight now_____

Highest weight_____ When?_____ For how long?_____
Lowest weight_____ When?_____ For how long?_____

7) How many times have you gained or lost at least 5 kg in your life?_____

8) How old were you when you first gained/lost weight?_____

9) What do you think caused you to gain/lose weight? _____

Appendix 5. International Physical Activity Questionnaire

Name:

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the **last 7 days**. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

Think about all the **vigorous** activities that you did in the **last 7 days**. **Vigorous** physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. Think *only* about those physical activities that you did for at least 10 minutes at a time.

1. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, digging, aerobics, or fast bicycling?
_____ **days per week**
No vigorous physical activities ***Skip to question 3***

2. How much time did you usually spend doing **vigorous** physical activities on one of those days?
_____ **hours per day**
_____ **minutes per day**
Don't know/Not sure

Think about all the **moderate** activities that you did in the **last 7 days**. **Moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal. Think only about those physical activities that you did for at least 10 minutes at a time.

3. During the **last 7 days**, on how many days did you do **moderate** physical activities like carrying light loads, bicycling at a regular pace, or doubles tennis? Do not include walking.
_____ **days per week**
No moderate physical activities ***Skip to question 5***

4. How much time did you usually spend doing **moderate** physical activities on one of those days?
_____ **hours per day**
_____ **minutes per day**
Don't know/Not sure

Think about the time you spent **walking** in the **last 7 days**. This includes at work and at home, walking to travel from place to place, and any other walking that you might do solely for recreation, sport, exercise, or leisure.

5. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time?
_____ **days per week**
No walking ***Skip to question 7***

6. How much time did you usually spend **walking** on one of those days?

_____ hours per day
_____ minutes per day
Don’t know/Not sure

The last question is about the time you spent **sitting** on weekdays during the **last 7 days**. Include time spent at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading, or sitting or lying down to watch television.

7. During the **last 7 days**, how much time did you spend **sitting** on a **week day**?
_____ hours per day
_____ minutes per day
Don’t know/Not sure

This is the end of the questionnaire, thank you for participating

Appendix 6. Food Diary Instructions

Time: Write down the time of day you ate the food.

Food/ drink item: Write down the type of food you ate or beverage you consumed. Be as specific as you can.

- Include sauces and gravies. Don't forget to write down "extras" such as salad dressings , mayonnaise, butter, sugar and ketchup.
- Remember to include all beverages, including water.
- Remember to include all supplements taken each day.

Details: Describe the food accurately and give as much information as possible

- Give brand names if possible
- Describe the form of the food (salted, unsalted, sweetened, fat-free)
- Describe how the food was purchased (fresh, frozen, canned) e.g. fresh pineapple vs. slices of pineapple canned- mainly applied for snacks (e.g. fruits)
- State any food fortified with additional nutrients
- Describe all the foods included in more complex dishes
- Include food preparation (e.g. boiling, roasting, baking, broiling, frying or steaming)- mainly applied for main meals (e.g. meat, chicken. pasta, potatoes, vegetables)
- Include the type and the amount of fat/ oil used
- Cut out nutrition and ingredient labels of packaged foods and attach to diet diary.
- If eating out, describe the food item well. Please record the name of popular fast food restaurants (e.g. Mac Donald's).
-

Amount eaten (g): Use the food weighing scale for measuring food

- ✓ To use the food weighing scale:
  1. Make sure the scale is on a flat surface like the kitchen counter.
  2. Remember to place the bowl on scale before switching on
  3. Press and release ON/OFF button. 88888/8 appears on display.
  4. Wait until display shows 0.
  5. Weigh cooked foods after cooking, and note on your food diary that you are recording cooked weight.
  6. Weigh several ingredients in the bowl, press ZERO to reset the display between each ingredient (for every new food you add). Make sure that food is put on the centre of the plate when you use a large plate.
  7. When you finish, press ON/OFF button to switch-off.
- ✓ You can weigh your food in the bowl on top of the scale, or you can use your plate. In this case put the plate on the scale before switching on and follow steps 3 to 7 as described above.
- ✓ To weigh fluids, use a cup instead of the bowl/plate. Follow steps 2-7.

Example 1: To weigh a slice of pizza on a plastic plate.

1) Place a plate on the scale before switching on.
2) Switch on the scale and wait until 0 is displayed.
3) Place the pizza slice on the scale and read the weight.
4) Record weight on the food diary.

Example 2: To weigh a piece of steak with rice on the bowl

1) Place the bowl on the scale before switching on.

2) Switch on the scale and wait until 0 is displayed.
3) Place the piece of steak on the scale and read the weight.
4) Record the weight of the piece of steak on the food diary.
5) Before weighing the rice, press 0.
6) Place the rice on the bowl and read the weight.
7) Record the weight of rice on the food diary.

Amount of leftovers (g): Use the scale for measuring leftovers

Example 1: To record the weight of uneaten food (like the crust of pizza)

1) Place the bowl on the scale before switching on.
2) Switch on the scale and wait until 0 is displayed.
3) Place the leftover pizza crust on the scale and read the weight.
4) Record the weight of the leftover pizza crust on the food diary form (amount left).

Example 2: To the record the weight of uneaten food- complex dishes (like mixed steak and rice)

1) Place the bowl on the scale before switching on.
2) Switch on the scale and wait until 0 is displayed.
3) Try to separate the steak from rice if you can.
4) Place the leftover piece of steak on the scale and read the weight.
5) Record the weight of the steak on the food diary form (amount left).
6) Before weighing the leftovers of rice, press 0.
7) Place the leftovers of rice on the bowl and read the weight.
8) Record the weight of rice left on the food diary form (amount left).

When you eat out:

1) Weigh your food if possible.
2) If weighing your food is not possible, use alternative methods (size of your palm for meat, fish and poultry; tablespoon for oils; toast slices for bread; CD size or cup size for pasta and rice; egg size for potatoes, cup size for salads).
3) Include the name of the restaurant.
4) Include nutritional information of the meal you consumed, if details are provided by the provider.

Cleaning the weighing scale:

- ✓ Bowl can be washed in hand or in a dishwasher.
- ✓ Clean the scale with slightly damp cloth. DO NOT immerse the scale in water or use chemical/abrasive cleaning agents.

Some basic rules to remember:

- ✓ Don’t change your eating habits while you are keeping your food diary.
- ✓ Tell the truth. There is nothing to be gained by trying to look good on these forms.
- ✓ Write everything down: Keep the scale and the form with you all day and write down everything you eat drink or eat.
- ✓ Do it now: record your eating as you go.
- ✓ Be specific: Include details and do not generalize

24 hour food record- Example

Time	Food/drink item	Details	Amount eaten (g)	Amount leftover (g)
08.05	Coffee	Strong and black	314	
08.05	Frosties cereal	Kelloggs	52	
08.05	Semi-skimmed milk		107	
08.10	Bread	Toasted, brown 2 slices	56	
08.10	Margarine	Lurpak	17	
08.10	Jam	Raspberry	13	
10.30	2 digestive biscuits	McVities	34	
10.30,14.20, 16.15	Coffee	3, strong and black	936	
12.30	Egg mayonnaise French stick (white)	From canteen	541	
12.30	Crisps	McCoys, ready salted	40	
12.30	Diluted orange squash drink		250ml	
16.15	Snickers		48	
17.45	Beef curry	Asda	450	34
17.45	Chips	Oven, McCain	207	
17.45	Tomato ketchup		23	
17.45	Diet Coca Cola		455	
20.45-22.30	Lager	Stella	4 pints	

Appendix 7. Food diary

Name:

Day: 1 2 3 (Circle one) **Date:**

Time	Food/ drink item	Details	Amount eaten (g)	Amount leftovers (g)

Appendix 8. Participant Information Sheet- Day to day Variability Study

"Repeatability of exercise energy expenditure measurements in physically active individuals by indirect calorimetry "

Brief Introduction

In previous research in our laboratory we have used indirect calorimetry to measure exercise energy expenditure (EEE). Indirect calorimetry measures oxygen consumption and carbon dioxide production which allows the estimation of EEE using formulas. It is a non-invasive, accurate and inexpensive method when the specific laboratory equipment required is available; characteristics that makes it one of the most commonly used method in determining EEE. In the proposed study, we aim to confirm that the indirect calorimetry system used in our laboratory is consistent in achieving identical results across multiple tests conducted under the same experimental conditions.

Study Requirements

To take part in this study you have to be 1) healthy, as determined by health history questionnaire, 2) aged between 18 and 40 years, 3) injury-free, 4) not on any current medication, 5) have a BMI= 18.5-30 $kg \cdot m^{-2}$, 6) able to follow verbal and written instructions and 7) in case of female participants, eumenorrheic or on oral contraceptives. Participants will be excluded if they are 1) breast-feeding 2) pregnant, 3) have a history of metabolic, heart, liver or kidney disease, diabetes, thyroid disorders or 4) have a BMI ($kg \cdot m^{-2}$)< 18.5 or >30.

Location

All the testing and analyses will be conducted in the Sports and Exercise Science, Biochemistry and Biosciences Laboratories at Nottingham Trent University.

Restrictions During Testing

You will be asked to record your food intake during Day 1 and replicate it for Days 2.

Testing Protocol

You will be asked to attend a preliminary session (P) and 2-day main experimental period (Day 1- Day 2). The preliminary assessment will be performed to establish inclusion criteria, take anthropometric measurements and determine your fitness level.

During Days 1-2 you will be asked to complete a standardized protocol for data collection consisting of 5 min of rest, followed by 2 sessions of 15 minutes running at 70% VO_{2max} separated by 5 min break on a flat treadmill. Exercise energy expenditure during the exercise will be measured using an indirect calorimetry system (a breath-by-breath system that requires wearing a facemask for expired gases collection and analysis). The experimental conditions will be standardised during Day 1-2. You will be asked to be tested the same day of the day for each of your sessions and at least 3 hours after your last meal. Food intake will be recorded for Day 1 and you will be asked to replicate it for Day 2, aiming at limiting the within participants' variations in exercise energy expenditure due to differences in food intake.

Potential Benefits to You

By participating in this study you will gain greater knowledge of measurement and monitoring exercise energy expenditure. Individually, you will improve your understanding of your individual fitness as you will receive individual VO2 max and heart rate results. Finally, if you decide to participate you will receive a full report indicating the results of the analysis of daily dietary intake.

Discomfort, inconveniences and risks of participating in the study

There are some risks and/or potential side effects associated with participating in this study. Capillary blood sampling through finger pricks may result in discomfort including mild pain, a sharp sensation or possibly some slights bruising of the fingers. Although it is extremely unlikely, high intensity exercise has been known to reveal unsuspected heart or circulation problems and very rarely these have had serious or fatal. The risk of adverse cardiovascular events in a young health population is minimal. However, should you feel unwell or any pain during the testing then please stop immediately and inform the experimenter. The assessments will be conducted under controlled conditions in facilities that are appropriate for each test in order to minimise the potential risks.

Voluntary Participation

Participation in this study is entirely voluntary meaning that you <u>do not</u> have to take part in

this study if you do not wish to. If at any point you decide to withdraw from the study your data will be destroyed. Whatever your decision, please be assured that it will not disadvantage you in any way.

Confidentiality

All the information collected from you for the study will be treated confidentially, and only the researchers named above will have access to it. The study results may be presented at a conference or in a scientific publication, but individual participants will not be identifiable in such a presentation.

Further Information and contacts

Please discuss the information above with others if you wish or ask us if there is anything that is not clear or if you would like more information.

Miss Maria Papageorgiou, email: maria.papageorgiou2012@my.ntu.ac.uk,
telephone: +44(0) 7762828659

Dr Craig Sale, email: craig.sale@ntu.ac.uk , telephone: +44 (0)115 848 3505

Dr Kirsty Elliott-Sale, email: kirsty.elliottsale@ntu.ac.uk, telephone: +44 (0)115 848 6338

Appendix 9. Informed Consent- Day to day Variability Study

Participant Statement of Consent to Participate in the Investigation Entitled:

"Repeatability of exercise energy expenditure measurements in physically active individuals by indirect calorimetry"

1) I, _____ **[*name]*** agree to partake as a participant in the above study.

2) I understand from the participant information sheet, which I have read in full, and from my discussion(s) with ***Maria Papageorgiou*** that this will involve me involve **my participation in exercise sessions *for 2 consecutive days.***

3) It has also been explained to me by *Maria Papageorgiou* that the risks and side effects which may result from my participation are as follows: slight bruising of my fingers due to capillary blood samples (via fingerpick) and minor muscle injury due to exercise session.

4) I confirm that I have had the opportunity to ask questions about the study and, where I have asked questions, these have been answered to my satisfaction.

5) I undertake to abide by University regulations and the advice of researchers regarding safety.

6) I am aware that I can withdraw my consent to participate in the procedure at any time and for any reason, without having to explain my withdrawal and that my personal data will be destroyed.

7) I understand that any personal information regarding me, gained through my participation in this study, will be treated as confidential and only handled by individuals relevant to the performance of the study and the storing of information thereafter. Where information concerning myself appears within published material, my identity will be kept anonymous.

8) I confirm that I have had the University's policy relating to the storage and subsequent destruction of sensitive information explained to me. I understand that sensitive information I have provided through my participation in this study, in the form of ***questionnaires and exercise records*** will be handled in accordance with this policy.

11) I confirm that I have completed the health questionnaire and know of no reason, medical or otherwise that would prevent me from partaking in this research.

Participant signature: Date:

Independent witness signature: Date:

Primary Researcher signature: Date:

Appendix 10. Participant Information Sheet-Studies 1 and 2

Information sheet for participants

Bone turnover in response to short-term energy restriction in men and women.

This study has been approved by the NTU Ethics Committee (Project ID Number:269)

Researchers: Maria Papageorgiou

Supervisors: Dr Craig Sale and Dr Kirsty Elliott-Sale

Background information

Physically active populations including athletes and military personnel are susceptible to stress fracture injuries. The consequences of such injuries vary from minor pain to severe lifelong disabilities. Sex differences in stress fracture epidemiology have been reported, with women being at higher risk. Although the cause of greater incidence of stress fractures in women is unknown and appears to be rather multi-factorial many studies emphasise the contribution of nutritional factors, dietary and exercise practices. Amongst them, energy availability (defined as the energy intake minus the energy expenditure) gains more and more scientific interest. Despite the fact that low energy availability is more common in women and there is evidence of negative effects of energy restriction on bone health in this gender there is little published information for men. The current study aims to explore the effects of low energy availability on bone responses in males and females. This will be achieved by administering controlled balanced and restricted energy availabilities through manipulation of both energy intake (diet) and energy expenditure (exercise).

Inclusion criteria

To take part in this study you have to be: 1) apparently healthy, 2) aged between 18 and 35 years, 3) injury-free, 4) not on any current medication 5) menstruate regularly (women), 6) participate in more than 3 hours of mild-moderate exercise per week 7) normal-weight 8) non-smokers 9) Caucasians and 10) have no history of major weight control problems or eating disorders. In addition, you should not have any of the following exclusion criteria:1) breast-feeding, 2) pregnancy, 3) women on oral contraceptive therapy at least in the previous 6 months, 4) women with amenorrhea, short, long or irregular menstrual cycles, 5) history of metabolic, heart, liver or kidney disease, diabetes, thyroid disorders, 6) congenital or acquired orthopaedic abnormalities, 7)experience of bone fracture in the previous 12 month and BMI< 18 or >25 kg/m^2 **.**

Study procedures

You will be required to complete two days of preliminary assessments and two, 9 d experimental periods. During preliminary measurements you will be required to fill out various forms (informed consent, health screen, training history, menstrual cycle questionnaire), to have body composition measured, perform a physical fitness test ($VO_{2\ max}$ test) and the main exercise protocol to determine your energy expenditure. The investigators of this study will provide your supervised transport to Derby University for a body scan to measure your body composition.

Over the first three days of each experimental period you will be asked to record your dietary intake and wear a monitor to record your physical activity. On the following 5 days you will be asked to perform daily approximately 1 h of exercise of 70% VO_{2max} in smaller sessions with break between them . At the same time, your dietary energy intake will be controlled either to achieve energy balance (close to your recommended calorie intake per day) or to lead to energy restriction (this will be like going on a diet for 5 days). The two testing periods will be carried out with approximately 30 days gap between testing. Blood samples will be collected and analysed for markers indicative of bone metabolism and hormones reflective of feeding and energy intake over the experimental protocol (see Figure 1).

Benefits

By participating in this study you will gain greater knowledge of how energy availability restriction affects bone health. Individually, you will improve your understanding of your individual fitness as you will receive individual $VO_{2\ max}$ and heart rate results. Furthermore, you will be provided the results of your individual assessment of body composition (body fat percentage, lean body mass) and bone mass. Finally, if you decide to participate you will receive a full report indicating the results of your assessment of daily dietary intake.

Discomfort, inconveniences and risks of participating in the study

There are some risks and/or potential side effects associated with participating in this study. For example, you may experience slight bruising due to the blood sample and exercise can result in injury and illness (particularly adverse cardiovascular events). However, the assessments will be conducted under controlled conditions in facilities that are appropriate for each test in order to minimise the potential for injury. The risk of adverse cardiovascular events in a young health population is minimal. However, should you feel unwell or any pain during the testing then please stop immediately and inform the experimenter. You may experience minor side-effects of energy restriction including dizziness, headaches and difficulties in concentrating. You will be supervised at all times whilst in the laboratory. Additionally, you will be closely monitored whilst not in the laboratory when under energy restriction. An experimenter will call you each day and/or check your condition by email. A buddy system will also be developed whenever possible to ensure your safety. Detailed instructions to seek medical advice in case of an emergency and/or to eat something if they feel unwell as a side-effect of energy restriction are provided (see leaflet). Furthermore, body composition measurement requires minor radiation exposure; amount equivalent to about one seventh of a chest x-ray, or 12 hours background radiation or the amount of radiation you would be exposed to walking across the University car park on a sunny day.

Voluntary Participation

Participation in this study is entirely voluntary meaning that you **do not** have to take part in this study if you do not wish to. If you do take part, you can withdraw at any time without having to give a reason. Whatever your decision, please be assured that it will not disadvantage you in any way.

Confidentiality

All the information collected from you for the study will be treated confidentially, and only the researchers named above will have access to it. The study results may be presented at a conference or in a scientific publication, but individual participants will not be identifiable in such a presentation.

Further Information

Please discuss the information above with others if you wish or ask us if there is anything that is not clear or if you would like more information.

Miss Maria Papageorgiou, email: maria.papageorgiou2012@my.ntu.ac.uk , telephone: +44(0) 7762828659

Dr Craig Sale, email: craig.sale@ntu.ac.uk , telephone: +44 (0)115 848 3505

Dr Kirsty Elliott-Sale, email: kirsty.elliottsale@ntu.ac.uk, telephone: +44 (0)115 848 6338

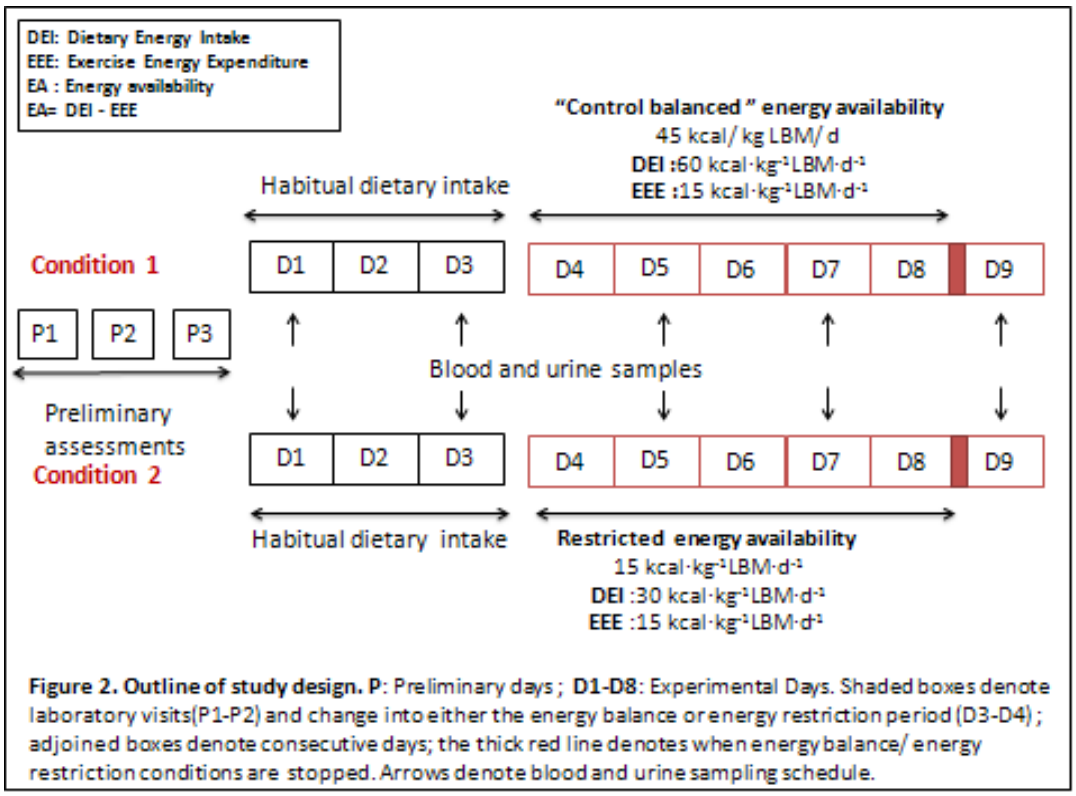


Figure 2. Outline of study design. P: Preliminary days ; **D1-D8**: Experimental Days. Shaded boxes denote laboratory visits(P1-P2) and change into either the energy balance or energy restriction period (D3-D4) ; adjoined boxes denote consecutive days; the thick red line denotes when energy balance/ energy restriction conditions are stopped. Arrows denote blood and urine sampling schedule.

Appendix 11. Informed Consent Form -Studies 1 and 2

Subject Statement of Consent to Participate in the Investigation Entitled:

"Bone turnover in response to short-term energy restriction in men and women."

1) I, _____*[your name]* agree to partake as a subject in the above study.

2) I understand from the participant information sheet, which I have read in full, and from my discussion(s) with Maria Papageorgiou that this will involve my participation in exercise sessions and either maintenance or restriction of my habitual dietary energy intake for 5 days during the experiment. Moreover, this study will involve my participation in body composition measurements at NTU or University of Derby. and fasting blood samples (20 ml) every second day during the 9 day experimental protocol, completed twice.

3) It has also been explained to me by *Maria Papageorgiou* that the risks and side effects which may result from my participation are as follows: slight bruising due to blood samples, minor muscle injury due to exercise session and minor side-effects of energy restriction.

4) I confirm that I have had the opportunity to ask questions about the study and, where I have asked questions, these have been answered to my satisfaction.

5) I undertake to abide by University regulations and the advice of researchers regarding safety.

6) I am aware that I can withdraw my consent to participate in the study at any time and for any reason, without having to explain my withdrawal.

7) I understand that any personal information regarding me, gained through my participation in this study, will be treated as confidential and only handled by individuals relevant to the performance of the study and the storing of information thereafter. Where information concerning myself appears within published material, my identity will be kept anonymous.

8) I confirm that I have had the University's policy relating to the storage and subsequent destruction of sensitive information explained to me. I understand that sensitive information I have provided through my participation in this study, in the form of *questionnaires and blood samples* will be handled in accordance with this policy.

10) I understand that as part of this study I will be consuming a multi-vitamin multi-mineral supplement.

11) I confirm that I have completed the health questionnaire and know of no reason, medical or otherwise that would prevent me from partaking in this research.

Subject signature: …… Date:_____

Independent witness signature: …… Date:_____

Primary Researcher signature: …….. Date:_____

Appendix 12. Participant Information Sheet- Study 3 and 4

Participant Information Sheet

Study Title: The effects of acute energy restriction by diet or exercise on bone metabolism, muscle function and cognition.

PART 1
We would like to invite you to volunteer in our research study. Before you decide we would like you to understand why the research is being done and what it would involve for you. **One of the study researchers will go through the information sheet with you and answer any questions you may have.** Please feel free to talk to others about the study if you wish. You may take as much time as you require to decide whether you would like to participate. This information sheet tells you the purpose of this study and what you will be required to do if you take. **Please ask if anything is not clear.**

Study description

Many populations restrict their energy intake (e.g. athletes, military recruits, obese patients on a weight loss programme) and recent research suggests that this may have negative consequences for bodily functions including bone health, muscle function and cognition. Although the unfavourable effects of energy restriction are becoming clearer, it is still not known if the method by which energy restriction is achieved (diet, exercise, or a combination of both) affects the response. Therefore, this study aims to examine if there are different responses to energy restriction when it is achieved by dietary restriction or increased energy expenditure (exercise) for bone metabolism, muscle function and cognition.

What is the purpose of the study?

The aim of this study is to investigate the effects of three days energy restriction achieved by diet or exercise on bone metabolism, muscle function and cognition.

Why have I been invited?

We are looking for healthy individuals who perform moderate-to-high intensity exercise at least 3 times per week and who would be capable and willing to complete three experimental conditions:

1. Three days controlled energy balance (~2000 kcal per day)
2. Three days dietary restriction (~600 kcal per day)
3. Three days increased energy expenditure (~2000 kcal per day with ~1400 kcal worth of exercise per day)

Do I have to take part?

It is entirely up to you to decide whether or not to join the study. We will describe the study and go through this information sheet, please feel free to ask

any questions if you are unsure of anything. If you agree to take part, we will then ask you to sign a consent form. You are free to withdraw at any time, without giving a reason.

What will happen to me if I take part?

You will be asked to complete a preliminary assessment (P) to establish suitability for the study , take body composition measurements (Dual-energy X-ray absorptiometry-DXA and ultrasound scan), undertake muscle and cognitive function tests and determine your fitness level (approximately 2 hours). Following this, you will be asked to record your habitual dietary intake and lifestyle physical activity for three days (H1-H3) before completing three experimental conditions. In each condition you will be asked to inform a member of the research team at the beginning of your next menstrual or oral contraceptive pill cycle (D1), undertake muscle function and cognitive function tests (D2 and D6), have a blood sample taken and undergo an experimental period (D3-5). In condition 1 you will consume an energy-balanced diet and perform no exercise. Condition 2 and 3 will induce the same level of energy restriction; condition 2 will be achieved by manipulating diet only, whereas condition 3 will be achieved by increasing exercise energy expenditure only. During condition 3 you will be required to visit the laboratory to complete exercise sessions lasting approximately 2.5 hours on each experimental day (D3-5). Blood samples will be collected twice; in the beginning (D2) and at the end (D6) on each condition (10 minutes procedure) and analysed for bone markers and endocrine factors. The three testing conditions will each be separated by approximately 25 days to include three menstrual cycles for eumenorrheic (regularly menstruating) participants and three pill taking cycles for oral contraceptive users. The whole testing period will take approximately 14 weeks (see Figure 1,2).

Expenses and Payments

You won't receive any payments for your participation in the study.

Food will be provided for the three experimental days (D3-5) in all three conditions.

What will I have to do?

During the preliminary assessment (P) we will explain the study to you, provide you an opportunity to ask questions and ask you for your written informed consent. You will then be asked to fill out questionnaires, undertake cognitive and muscle function tests, perform a fitness test and undergo body composition scans (this will be in the form of Dual-energy X-ray absorptiometry (DXA), which is used for making measurements of body composition and bone mineral density and an ultrasound scan used to measure the diameter of a muscle in your hand). For the DXA scan you will be asked to remove your shoes and any metal objects and lay on your back on the bed of the scanner. An x-ray beam will then pass slowly over your whole body for approximately 8 minutes. You will not feel any sensation from this beam.

During the habitual assessment (H1-H3) you will be asked to record your habitual dietary intake and lifestyle physical activity. You will then be asked to complete three experimental conditions. In each condition you will be asked to notify the experimenter at the beginning of your menstrual or oral contraceptive cycle (D1). On the next day (D2), you will be asked to visit the laboratory for a blood sample and to complete baseline muscle and cognitive function tests. The following three days of the protocol (D3-D5) will be the experimental period. Over the three experimental days you will undertake condition 1, condition 2 or

condition 3. In condition 1 you will consume an energy balanced diet and be asked to refrain from any exercise. In condition 2 you will be asked to refrain from any exercise and maintain a low-calorie diet for three days. During this condition you will be asked to consume a multi-vitamin multi-mineral supplement to ensure adequate micronutrient intake. In condition 3 you will provided with a normal diet and be asked to complete exercise sessions at 70% of your maximal oxygen uptake in two sessions per day (approximately 1 hour in the morning- 1 hour in the afternoon; each 1 hour will be performed in 15 minutes sessions with 5 minutes break between them) for three days. Blood samples will be collected and muscle and cognitive performance tests will be conducted at the end of the protocol (D6). All blood samples will be taken from a vein in the forearm by an experienced phlebotomist.

What are the possible disadvantages and risks of taking part?

During the process of being scanned you will receive a small dose of radiation (30 μSv), which is very small, compared to other X-ray procedures and is the equivalent to the additional cosmic radiation dose received from a flight from the UK to Spain. Blood sampling may result in discomfort including mild pain, a sharp sensation or possibly some bruising. However, all procedures will be conducted under controlled conditions in facilities that are appropriate for each test in order to minimise the potential for injury. However, should you feel unwell or any pain during the testing then please stop immediately and inform the experimenter. There is always a risk for exercise to result in adverse cardiovascular events or injury, however this is unlikely as the criteria for participation in this study stipulate that you must regularly exercise and will therefore be accustomed to exercising. You may experience minor side-effects of energy restriction including dizziness, headaches and difficulties in concentrating. You will be supervised at all times whilst in the laboratory and will be contacted by phone each morning and evening during the energy restriction conditions to confirm you are well. Detailed instructions to seek medical advice in case of an emergency and/or to eat something if you feel unwell as a side-effect of energy restriction are provided (Energy restriction emergency instructions) .

What are the possible benefits of taking part?

We cannot promise that the study will help you, but the information we get from this study will contribute to the available knowledge concerning the effects of energy restriction on bone health, muscle function and cognitive function in physically active and obese/overweight populations. You will improve your understanding of your individual fitness, as you will receive individual $VO_{2\ max}$ and heart rate results. Furthermore, you will be provided the results of your individual assessment of body composition (body fat percentage, lean body mass) and bone mineral density (BMD). Finally, if you decide to participate you will receive a full report indicating the results of your assessment of daily dietary intake.

What happens when the research study stops?

The information from the study (fitness test, body composition and bone mass measurement and dietary analysis) will be provided as feedback to you at the end of your participation.

What if there is a problem?

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The detailed information on this is given in Part 2.

Will my taking part in this study be kept confidential?

Yes. We will follow ethical and legal practice and all information about you will be handled in confidence. The details are included in Part 2.

If the information in Part 1 has interested you and you are considering participation, please read the additional information in Part 2 before making any decision.

PART 2

What if relevant new information becomes available?

If new information comes available that is applicable to the safety of the study we will inform you of this information. If the study is stopped for any reason, you to be informed with regard to the reasons.

What will happen if I don't want to carry on with the study?

You are free to withdraw from the study at any point without providing a reason and without consequence. If you withdraw from the study, we will ask you if you give us permission to use any data collected from you until that point. If you do not consent to this, we will delete all data pertaining to you that is stored on computers or hard copies and destroy any samples collected from you.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions using the contact details at the end of this information sheet. If you remain unhappy and wish to complain formally, you can do this by contacting Nottingham Trent University's technical manager, Mark Cosgrove Tel: 0115 8486691, who is independent of the research program and will take you through the complaints procedure.

Will my taking part in this study be kept confidential?

Yes. We will follow ethical and legal practice and all information about you will be handled in confidence. All information will be coded and stored securely. Any information about you which leaves the University will have your name and address removed so that you cannot be recognised (e.g., in case of a publication). All data will be used for analysis in the present study. All data will be destroyed no later than 5 years post the award of a PhD for the research students involved in this study.

Involvement of the General Practitioner/Family doctor (GP)

You may wish is seek advice from your GP, however we will not inform your GP about your participation in the study.

What will happen to any samples I give?

Your blood samples will be collected in coded tubes and stored in freezers housed within the Department of Sport Science laboratories. Your samples will only be identifiable to the research team. Your data from the body scan, dietary analysis, physical activity records and questionnaires will be stored in a locked cabinet or on a password protected university computer. Your samples will be stored for no later than 5 years post the award of a PhD for the research students involved in this study. Your samples will be analysed at Nottingham Trent University and at the University of East Anglia on a collaborative basis.

What will happen to the results of the research study?

The results of the study will be provided as feedback to you and also published in peer reviewed academic journals. Information will be provided as to the location of the publication when this information is known. You will not be identified in any report or publication.

Who is organising and funding the research?

The research is organised and funded by Nottingham Trent University.

Who has reviewed the study?

The research is looked at by an independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given favourable opinion by NRES Committee East Midlands - Derby (approval number: 154899) and Nottingham Trent University's Research Ethics Committee (Humans).

Further information

1. General information about research: we would be happy to provide you with further details about this and other research projects that we are working on. Please contact any member of the research team using the contact details below.

2. Specific information about this research project; if you would like more written information about this study we are happy to provide this or if you would like to talk about any aspects of this study then please feel free to contact any member of the research team using the contact details below.

3. Advice as to whether you should participate: we would be happy to discuss your suitability to participate in this study or alternatively please feel free to seek advice from others outside of the research team and/or from a healthcare professional.

4. Who you should approach if unhappy with the study: if you wish to speak with someone outside of the research team, please contact Mark Cosgrove Tel: 0115

8486691, who is independent of the research program and will take you through the complaints procedure.

Contact Details:

Investigator:
Maria Papageorgiou, MSc
Email: Maria.papageorgiou2012@my.ntu.ac.uk
Postgraduate Researcher
Biomedical, Life and Health Sciences Research Centre
School of Science and Technology,
Nottingham Trent University,
Erasmus Darwin Building,
Clifton Lane,
Nottingham, UK.
NG11 8NS.
Tel: 0115 8483820

Investigator:
Daniel Martin, MRes
Email: Daniel.martin@ntu.ac.uk
Academic Associate in Sport Science
Biomedical, Life and Health Sciences Research Centre
School of Science and Technology,
Nottingham Trent University,
Erasmus Darwin Building,
Clifton Lane,
Nottingham, UK.
NG11 8NS.
Tel: 0115 8483820

Academic Supervisor:
Dr Craig Sale, MSc, PhD
Email: Craig.Sale@ntu.ac.uk
Reader in Applied Physiology
Biomedical, Life and Health Sciences Research Centre
School of Science and Technology,
Nottingham Trent University,
Erasmus Darwin Building,
Clifton Lane,
Nottingham, UK.
NG11 8NS.
Tel: 0115 8483505

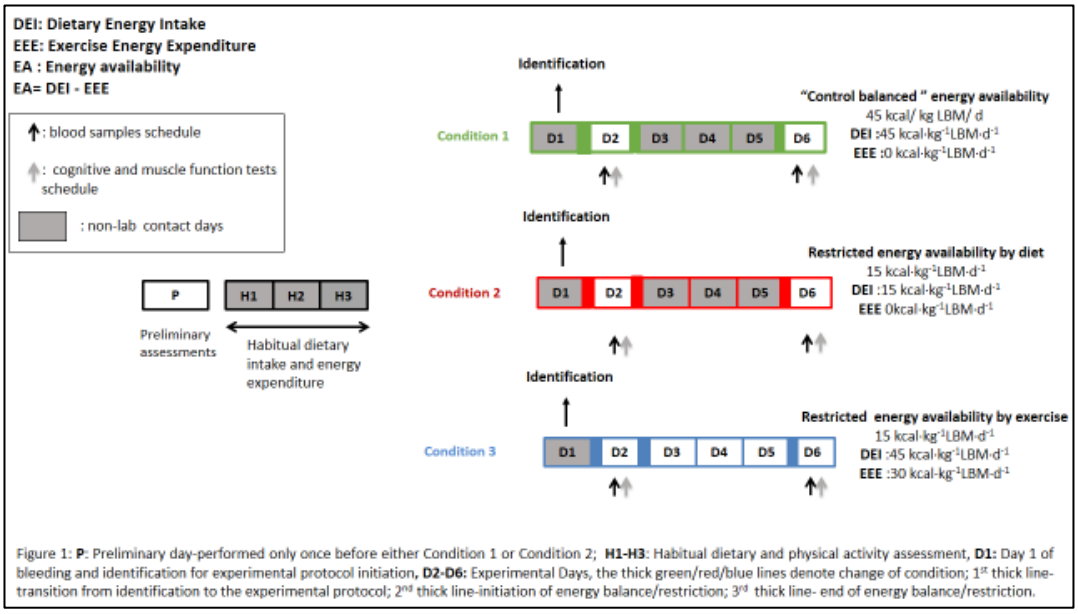


Figure 1: **P**: Preliminary day-performed only once before either Condition 1 or Condition 2; **H1-H3**: Habitual dietary and physical activity assessment, **D1:** Day 1 of bleeding and identification for experimental protocol initiation, **D2-D6:** Experimental Days, the thick green/red/blue lines denote change of condition; 1st thick line-transition from identification to the experimental protocol; 2nd thick line-initiation of energy balance/restriction; 3rd thick line- end of energy balance/restriction.

Figure 1: Outline of study design

Preliminary assessments: Approximately 2 h (questionnaires, body composition assessment, fitness test, performance test familiarisation)

Habitual dietary intake and physical activity assessment (H1-H3)

Condition 1: Controlled, energy balanced diet	**Condition 2: Dietary restriction**	**Condition 3: Exercise**
D1:-	D1:-	D1:-
D2: 5 minutes blood sample + 30 minutes cognitive and muscle function test	D2: 5 minutes blood sample + 30 minutes cognitive and muscle function test	D2: 5 minutes blood sample + 30 minutes cognitive and muscle function test
D3: -	D3: -	D3: ~ 1-1.5 h exercise in the morning + ~ 1-1.5 h exercise in the afternoon evening
D4: -	D4: -	D4: ~ 1-1.5 h exercise in the morning + ~ 1-1.5 h exercise in the afternoon evening
D5: -	D5: -	D5: ~ 1-1.5 h exercise in the morning + ~ 1-1.5 h exercise in the afternoon evening
D6: 5 minutes blood sample + 30 minutes cognitive and muscle function test	D6: 5 minutes blood sample + 30 minutes cognitive and muscle function test	D6: 5 minutes blood sample + 30 minutes cognitive and muscle function test
Total visits: 3 lab visits, **Total duration:** ~ 1 h 35 minutes	**Total visits:** 3 lab visits, **Total duration:** ~ 1 h 35 minutes	**Total visits:** 9 lab visits, **Total duration:** ~9 h 5 min

Figure 2: Representation of visits to the laboratory throughout the study

Figure 2: Time commitment required by each experimental condition

Appendix 13. Informed Consent - Studies 3 and 4

Subject Statement of Consent to Participate in the Investigation Entitled:

"Effects of short term energy restriction achieved by diet or exercise on bone turnover, muscle function and cognition."

1) I, _____*[your name]* agree to partake as a subject in the above study.

2) I understand from the participant information sheet, which I have read in full, and from my discussion(s) with *Maria Papageorgiou and/or Dan Martin* that this will involve my participation in energy restriction for 3 days on two different occasions (exercise or diet) and energy balance for 3 days on one occasion. Moreover, this study will involve my participation in body composition measurements, muscle force production and cognitive function tests and fasting blood samples (20 ml on 6 occasions) during the study.

3) It has also been explained to me by *Maria Papageorgiou and/or Dan Martin* that the risks and side effects which may result from my participation are as follows: slight bruising due to blood samples, muscle injury due to exercise sessions and side-effects of energy restriction.

4) I confirm that I have had the opportunity to ask questions about the study and, where I have asked questions, these have been answered to my satisfaction.

5) I undertake to abide by University regulations and the advice of researchers regarding safety.

6) I am aware that I can withdraw my consent to participate in the study at any time and for any reason, without having to explain my withdrawal.

7) I understand that any personal information regarding me, gained through my participation in this study, will be treated as confidential and only handled by individuals relevant to the performance of the study and the storing of information thereafter. Where information concerning myself appears within published material, my identity will be kept anonymous.

8) I confirm that I have had the University's policy relating to the storage and subsequent destruction of sensitive information explained to me. I understand that sensitive information I have provided through my participation in this study, in the form of questionnaires and blood samples will be handled in accordance with this policy.

10) I understand that as part of this study I will be consuming a multi-vitamin multi-mineral supplement.

11) I confirm that I have completed the health questionnaire and know of no reason, medical or otherwise that would prevent me from partaking in this research.

Subject signature: …… Date:_____

Independent witness signature: …… Date:_____

Primary Researcher signature: ……. Date:_____

Appendix 14. Experimental Diets for Studies 3 & 4

Menu 1	CON, E-RES	D-RES
Milk	570	190
Weetabix	80	26
Jellypot	115	115
Potato and Leek Soup	495	165
Pitta bread	110	36
Bannanas	210	73
Tuna	160	53
Mayonnaise	51	17
Salad	70	70
White Pitta	100	33

Menu 2	CON, E-RES	D-RES
Milk	500	166
Weetabix	80	27
Jellypot	115	115
Tomato soup	500	166
Pitta bread	110	36
Bananas	230	76
Roast Chicken Breast	180	60
Mayonnaise	40	13
Salad	70	70
White Pitta	90	30
French dressing	50	16

Menu 3	CON, E-RES	D-RES
Bran Flakes	90	30
Milk	510	170
Jellypot	115	115
Potato and Leek Soup	500	166
Pitta bread	100	33
Bananas	220	73
Tikka Chicken Breast	180	60
Mayonnaise	48	16
Salad	70	70
Pitta bread	90	30
French dressing	45	15

Menu 4	CON, E-RES	D-RES
Bran Flakes	90	30
Milk	500	166
Jellypot	115	115
Tomato soup	500	166
Pitta bread	100	33
Bananas	220	73
BBQ Chicken Breast	160	53
Mayonnaise	39	13
Salad	70	70
Pitta bread	100	33
French dressing	45	15

Appendix 15. Combined data for combined OCP users and EU women

Table 1. Participant characteristics- combined data for combined OCP users and EU women

	Total (n=20)
Demographics	
Age (y)	25.5 (3.7)
Height (m)	1.65 (0.05)
Body mass (kg)	59.58 (6.0)
BMI (kg ·m^2)	21.8 (2.0)
Body composition	
Body fat (%)	27.8 (4.7)
Lean body mass (kg)	41.2 (3.6)
Fat free mass (kg)	43.5 (3.4)
BMD (g·cm^2)	1.18 (0.08)
Training characteristics	
$VO_{2\ max}$ (ml·kg^{-1}·min^{-1})	48.9 (5.3)
$VO_{2\ max}$ (ml·kg LBM^{-1}·min^{-1})	70.4 (6.1)
Physical activity(MET-min·week^{-1})	4068 (1921)
Dietary and EE characteristics	
Habitual DEI (kcal·d^{-1})[1]	2076 (372)
Lifestyle EE (kcal·d^{-1})	398 (126)
24-hour EE(kcal· kg ·d^{-1})[1]	2015 (183)

Values are expressed as means (1SD).
[1]Analysis performed in 17 participants with complete data.
BMI: Body Mass Index; BMD: Bone mineral density: VO_{2max}: Maximum oxygen uptake; DEI: Dietary Energy Intake; EE: Energy Expenditure; RMR: Resting Metabolic Rate; LBM: Lean Body Mass; MET: Metabolic equivalents; OCP: Combine Oral Contraceptive Pill Users; EU: Eumenorrheic.

Table 2. Baseline concentrations of BTMs, markers of calcium metabolism and regulatory hormones-combined data for combined OCP users and EU women. Data are presented as the mean BASE values prior to CON, D-RES and E-RES in EU women and combined OCP users together.

	Total (n=20)
BTMs	
β-CTX (µg·L^{-1})	0.44 (0.17)
P1NP (µg·L^{-1})	48.2 (15.2)
BT ratio	1.14 (0.25)
Markers of calcium metabolism	
PTH (pg·mL^{-1})	3.9 (1.0)
ACa (mmol·L^{-1})	2.31 (0.06)
Mg (mmol·L^{-1})	0.80 (0.03)
PO_4 (mmol·L^{-1})	1.28 (0.10)
Regulatory hormones	
IGF-1 (mmol·L^{-1})	203.4 (44.5)
T_3 (mmol·L^{-1})	1.66 (0.33)
Leptin (ng·mL^{-1})	7.4 (3.3)
Insulin (pmol·L^{-1})	39.7 (16.3)

Values are expressed as means (1SD).
β-CTX: C-terminal telopeptides of type I collagen; P1N: Amino-terminal Pro-peptides of Type 1 Procollagen; BT ratio: Bone turnover ratio; 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; OCP: Oral contraceptive pill; EU: Eumenorrheic; CON: Controlled; D-RES: Diet-induced restricted trial; E-RES: Exercise-induced restricted trial.

Table 3. BTMs expressed as concentrations, percentage change from BASE and AUC in CON, D-RES and E-RES trials in total sample of combined OCP users and EU women. Values at D2 were used as BASE prior to each experimental condition.

	Total (n=20)					
	CON		**D-RES**		**E-RES**	
	BASE	**D6**	**BASE**	**D6**	**BASE**	**D6**
β-CTX						
µg·L^{-1}	0.44 (0.18)	0.45 (0.17)	0.45 (0.16)	0.47 (0.16)	0.44 (0.19)	0.43 (0.19)
%BASE change		3.9 (16.1)		7.5 (20.0)		3.8 (34.3)
AUC(%BASE x d)[1]		7.8 (32.2)		14.9 (40.0)		7.7 (68.6)
P1NP						
µg·L^{-1}	48.8 (16.2)	46.1 (11.9)	46.8 (15.8)	41.6 (10.7)	48.9 (15.6)	46.1 (14.7)
%BASE change		-3.0 (12.0)		-6.4 (26.2)		-3.9 (21.9)
AUC(%BASE x d)[1]		-6.1 (24.0)		-12.8 (52.3)		-7.8 (43.9)
BT ratio						
-	1.17 (0.29)	1.08 (0.19)	1.08 (0.29)	0.90 (0.16)	1.18 (0.35)	1.10 (0.23)
%BASE change		-4.5 (18.6)		-10.5 (27.6)		1.0 (40.0)
AUC (%BASE x d)[1]		-9.0 (37.2)		-20.9 (55.2)		1.9 (80.0)

Values are expressed as means (1SD);
[1]AUC calculated for each experimental condition from BASE to D6.
[*]denotes a significant difference from BASE at the same condition (P<0.05)
β-CTX: C-terminal telopeptides of type I collagen; P1NP: Amino-terminal Pro-peptides of Type 1 Procollagen; BT ratio: Bone turnover ratio; CON: Controlled trial; AUC: Area under the curve; D-RES: Diet-induced E-RES:Exercise-Induced Restricted trial; BASE: Baseline; OCP: Oral contraceptive pill; EU: Eumenorreic.

Table 4. Markers of calcium metabolism expressed as concentrations, percentage change from BASE and AUC in CON, D-RES and E-RES trials for total sample of combined OCP users and EU women. Values at D2 were used as BASE prior to each experimental condition.

	Total (n=20)					
	CON		**D-RES**		**E-RES**	
	BASE	**D6**	**BASE**	**D6**	**BASE**	**D6**
PTH						
$pg \cdot mL^{-1}$	3.70 (1.09)	3.41 (0.64)	3.96 (1.50)	3.44 (0.77)	4.13 (1.27)	3.58 (0.81)
%BASE change		-3.1 (22.8)		-5.5 (28.2)		-8.0 (28.3)
AUC (%BASE x d)[1]		-6.2 (45.6)		-11.1 (56.3)		-16.0 (56.6)
ACa						
$mmol \cdot L^{-1}$	2.32 (0.07)	2.32 (0.07)	2.29 (0.06)	2.32 (0.09)	2.30 (0.07)	2.28 (0.06) *,**,†
%BASE change		0.1 (2.0)		1.1 (2.3)		-1.0 (2.0)
AUC (%BASE x d)[1]		0.3 (3.9)		2.1 (4.6)		-1.9 (4.1)†
Mg						
$mmol \cdot L^{-1}$	0.81 (0.04)	0.81 (0.05)	0.80 (0.03)	0.81 (0.05)	0.80 (0.05)	0.81 (0.04)
%BASE change		-0.2 (2.9)		1.0 (5.3)		1.4 (5.5)
AUC (%BASE x d)[1]		0.4 (5.7)		2.0 (10.7)		2.8 (10.9)
PO_4						
$mmol \cdot L^{-1}$	1.28 (0.12)	1.23 (0.13)	1.27 (0.13)	1.25 (0.12)	1.29 (0.13)	1.17 (0.09)*
%BASE change		-3.7 (10.0)		-1.0 (11.5)		-8.6 (9.1)
AUC (%BASE x d)[1]		-7.3 (20.1)		2.0 (23.0)		-17.1 (18.2)

Values are expressed as means (1SD).

[1]AUC calculated for each experimental condition from BASE to D6.

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

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

†denotes a significant difference from E-RES at the same timepoint ($P<0.05$).

PTH: Parathyroid hormone; Mg: Magnesium; ACa: Albumin adjusted Calcium; PO_4: Phosphate; BASE: Baseline; AUC: Area under the curve; CON, Controlled trial; D-RES, Diet-Induced Restricted trial; E-RES: Exercise-Induced Restricted trial; OCP: Oral contraceptive pill; EU: Eumenorrheic.

Table 5. Regulatory hormones expressed as concentrations, percentage change from BASE and AUC in CON, D-RES and E-RES trials for total sample of combined OCP users and EU women. Values at D2 were used as BASE prior to each experimental condition.

	Total (n=20)					
	CON		**D-RES**		**E-RES**	
	BASE	**D6**	**BASE**	**D6**	**BASE**	**D6**
T_3						
mmol·L^{-1}	1.65 (0.35)	1.67 (0.18)	1.67 (0.34)	1.45 (0.33) *,**,†	1.65 (0.33)	1.62 (0.40)
%BASE change		2.0 (14.0)		13.2 (9.9)		1.7 (13.0)
AUC (%BASE x d)[1]		3.9 (28.0)		-26.5 (19.9) **,†		-3.4 (26.0)
IGF-1						
mmol·L^{-1}	200.7 (42.9)	205.3 (47.8)	204.8 (47.1)	177.1 (34.3) *,**,†	204.7 (55.2)	165.4 (41.3) *,**
%BASE change		2.9 (16.3)		-12.7 (8.6)		-17.1 (17.3)
AUC (%BASE x d)[1]		5.9 (32.6)		-25.4 (17.1) **		-34.4 (34.6) **
Leptin[1]						
ng·mL^{-1}	7.2 (3.8)	5.1 (2.8) *	6.9 (3.2)	2.7 (1.7) *,**	7.0 (4.2)	3.2 (2.4) *,**
%BASE change		-26.0 (21.0)		-58.8 (16.5)		-53.5 (22.4)
AUC (%BASE x d)[1]		-51.9 (42.0)		-119.6 (32.9) **		-107.0 (44.7) **
Insulin						
ng·mL^{-1}	40.9 (30.8)	36.6 (18.5)	35.9 (13.1)	28.2 (13.4)	42.5 (27.7)	23.2 (10.1) *,**
%BASE change		2.2 (48.2)		-13.2 (50.8)		-29.5 (43.3)
AUC (%BASE x d)[1]		4.3 (96.3)		-26.4 (101.7)		-59.1 (86.7)

Values are expressed as means (1SD).

[1]AUC calculated for each experimental condition from BASE to Day 6.

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

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

†denotes a significant difference from E-RES at the same timepoint (P<0.05).

T_3: Triiodothyronine; IGF-1: Insulin-like growth factor 1; BASE: Baseline; AUC: Area under the curve; CON: Controlled trial; D-RES, Diet-induced restricted trial; E-RES: Exercise-induced restricted trial; OCP: Oral contraceptive pill; EU: Eumenorrheic.