

THE INFLUENCE OF DIET AND
NUTRITION ON BONE
METABOLISM IN ENDURANCE
ATHLETES

By

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ABSTRACT

Both accelerated and suppressed bone remodelling can lead to the development of a stress fracture injury. A stress fracture injury can threaten an athlete's performance by causing months of missed training time if a stress fracture is sustained during a crucial phase of the season. This thesis presents a series of studies that investigated bone metabolism in endurance athletes and potential ways to improve bone health and reduce the risk of stress fracture injury.

Triathletes are endurance athletes that anecdotally have a high incidence of stress fracture injury, but there is limited research into bone health in these athletes. Therefore, the first two studies in this thesis investigated bone metabolism in a group of elite British triathletes during off-season and pre-competition training. The results showed that elite triathletes had elevated bone turnover at both phases of the season, although this was highest during off-season training. The high bone turnover may be related to large training volumes, low energy intakes and high dermal calcium losses in the sweat.

Given the potential contribution of high dermal calcium losses to the disruption of calcium homeostasis and the different rates of losses in different types of training sessions, the timing of calcium ingestion around training sessions may be more important than total calcium intakes throughout the day. As such, the third study in this thesis investigated the mechanism and timeframe of Parathyroid hormone (PTH) and calcium regulation during exercise and recovery. The results showed that PTH secretion was controlled by a combination of changes in ionised calcium (Ca^{2+}) and phosphate (PO_4) and that the mechanism might be different during exercise and recovery. Taken together these results advocate the use of pre-exercise calcium supplementation, which may prevent the disruption of calcium homeostasis and attenuate the PTH and bone resorption response to intense exercise, although further research is required before this can be implemented in elite triathletes.

The large training volumes performed by elite triathletes, meant that daily energy expenditures and energy requirements were high, although consuming almost 6,000 kcal·d⁻¹ was difficult when three or four training sessions were regularly performed each day. Therefore, a practical nutritional intervention was needed to help triathletes ingest some of the required nutrients. The fourth study in this thesis investigated the effect of a post-exercise carbohydrate and protein (CHO+PRO) recovery solution on the bone metabolism response to an intense running bout. The results showed that consuming a CHO+PRO recovery solution immediately after exercise created a more positive bone turnover balance in the acute recovery period from exercise, by suppressing bone resorption and increasing bone formation. Further research is required to explore the long-term effects of post-exercise suppression of bone resorption.

This thesis had direct impact on elite British triathletes, by influencing athlete behaviour and nutritional practices in the daily training environment. The research has also influenced British Triathlon coaches and sports science and medicine staff by increasing the importance placed on bone health and by providing information that will allow training and nutritional practices to be improved or altered to promote a more anabolic environment for bone.

Key words: Bone, Bone Turnover, Bone metabolism, Bone Injury, Stress Fracture Injury, Endurance, Athlete, Triathlete, Training, Nutrition, Parathyroid Hormone, Calcium, Carbohydrate, Protein.

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DEFINITIONS OF TERMS

Elite athlete	Full-time professional athlete, competing at international and/or national level
Sub-elite athlete	Part-time professional athlete, competing at national and/or regional level
Trained athlete	Non-professional athlete, competing at regional and/or county level
Recreationally active	Non-athlete, competing at local club level or not competing, but training at least 3 times per week
Endurance athlete	An athlete competing in sports such as triathlon (sprint distance to Iron Man distance), distance running (5,000 m to ultra-distance races such as the Marathon des Sables), road cycling, race walking, trail or mountain running.

LIST OF ABBREVIATIONS

ACa	albumin-adjusted calcium
ANOVA	analysis of variance
BALP	bone alkaline phosphatase
BMC	bone mineral content
BMD	bone mineral density
(β) CTX	carboxyterminal cross-linking telopeptide of type 1 collagen
Ca ²⁺	ionised calcium
CHO	carbohydrate
CHO+PRO	carbohydrate and protein
CV	coefficient of variation
DF	delayed feeding
DLW	doubly labelled water
DXA	dual-energy X-ray absorptiometry
DPD	deoxypyridinoline
ECLIA	electro-chemiluminescence immuno assay
ELISA	enzyme-linked immunosorbent assay
EB	easy bike
ER	easy run
GIP	glucose-dependent insulintropic peptide
GLP-1	glucagon-like peptide-1
GLP-2	glucagon-like peptide-2
HB	hard bike
HR	hard run

ICTP	carboxyterminal cross-linked telopeptide of type 1 procollagen
IF	immediate feeding
IGF-1	insulin growth factor-1
IL	interleukin
IRMS	isotope-ratio mass spectrometry
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
NTX	aminoterminal cross-linking telopeptide of type 1 collagen
OC	osteocalcin
OPG	osteoprotegerin
P1CP	carboxyterminal propeptide of type 1 procollagen
P1NP	aminoterminal propeptide of type 1 procollagen
PLA	placebo
PO ₄	phosphate
pREE	predicted resting energy expenditure
PRO	protein
PTH	parathyroid hormone
PYD	pyridinoline
QCT	quantitative computed tomography
RA	recreationally active individuals
RANK	receptor activator of nuclear factor kappa-B
RANKL	receptor activator of nuclear factor kappa-B ligand
REE	resting energy expenditure
RED-S	relative energy deficiency in sport
RMR	resting metabolic rate

TEE	total energy expenditure
TRAP5b	tartrate-resistant acid phosphatase (isoenzyme 5b)
The triad	the female athlete triad
Total 25(OH)D	total 25-hydroxy vitamin D
T3	3,3,5-triiodothyronine
u-PYD	urinary pyridinoline
VO _{2max}	maximal oxygen consumption
V-SMOW	Vienna-standard mean ocean water
1,25(OH) ₂ D ₃	1,25, dihydroxyvitamin D

CHAPTER 1: INTRODUCTION

Bone mass is gained and lost throughout an individual's lifetime, due to changes in the relative amounts of bone formation and bone resorption (Weaver, 2000; Bachrach, 2001; Cashman, 2007). These changes are reflected in longer term alterations to bone mineral density (BMD) and bone structure, which are measured through bone scans such as dual-energy X-ray absorptiometry (DXA) or quantitative computed tomography (QCT), or in acute changes in biochemical markers of bone turnover, which are measured in blood or urine. For example, the menopause causes an increase in bone turnover, with greater increases in bone resorption than bone formation, leading to a loss of bone mass (Garnero *et al.*, 1996a).

Bone remodelling involves the sequential and coordinated actions of osteoclasts and osteoblasts, where the processes of bone formation and bone resorption occur in a cycle (Stewart and Hannon, 2000; Hadjidakis and Androulakis, 2006). Bone remodelling has three essential roles; it can access stores of calcium and phosphate (PO_4) from the bone, that are released into the serum when needed, maintaining homeostasis of these essential minerals, and it provides a mechanism for the skeleton to both adapt to mechanical loading and repair damage caused by mechanical loading (Parfitt, 1994; Parfitt *et al.*, 2000; Shaffler and Jepson, 2000; Burr, 2002; Frost, 2003). The amount of bone removed and the amount formed should be similar during adulthood, meaning that the total quantity of bone remains constant under healthy conditions (Currey, 2003). There are various factors that affect bone remodelling and the balance between bone resorption and bone formation, including; age, fractures, drugs and disease, exercise, menstrual status, systemic and reproductive hormones and diet (Bennell *et al.*, 1996a; Vasikaran *et al.*, 2011a). An imbalance in bone remodelling, where the processes of bone resorption and bone formation become uncoupled and shift in favour of increased bone resorption, can cause bone loss, and this uncoupling has been implicated in the formation of stress fracture injuries (Schaffler *et al.*, 1990).

A stress fracture can be defined as a partial or complete fracture of a bone that is a result of repeated stress applied to the bone that is less than the force required to fracture a bone in a single application (Martin and McCulloch, 1987). The development of a stress fracture involves repetitive loading, bone fatigue, microdamage accumulation and increased bone remodelling (Grimston and Zernicke,

1993; Bennell *et al.*, 1996a). Based on the studies by Straus (1932), Roberts *et al.* (1939), Murguia *et al.* (1988) and Mori and Burr (1993), Bennell *et al.* (1996a) proposed two hypotheses that may explain the development of a stress fracture. The first is the Primary Microdamage Hypothesis, which suggests that microdamage occurs first due to repetitive loading and a remodelling response is initiated in order to repair the microdamage that has accumulated. The second is the Primary Remodelling Hypothesis, which suggests that accelerated bone remodelling caused by diet, exercise or hormonal factors leads to weakened sites on the bone surface and when subsequent bone strain occurs, microdamage may accumulate at these already weakened areas. In both cases, if microdamage accumulation exceeds repair by the remodelling process, a stress fracture may develop.

The risk of stress fracture injury is greater in athletic populations due to the high amounts of mechanical loading experienced and this varies amongst athletes of different sports, as does BMD and a low BMD is a risk factor for stress fracture injury development (Carbon *et al.*, 1990; Myburgh *et al.*, Bennell *et al.*, 1996; Wentz *et al.*, 2012). There are groups of athletes that generally have high BMDs, such as weight lifters and games players (Karlsson *et al.*, 1993; Bennell *et al.*, 1997; Calbet *et al.*, 2001; Egan *et al.*, 2006), and groups that generally have low BMDs, such as road cyclists (Maïmoun *et al.*, 2004b; Rector *et al.*, 2008; Medelli *et al.*, 2009a; Scofield and Hecht, 2012), dancers (Kahn *et al.*, 1996), swimmers (Nikander *et al.*, 2010; Gomez-Bruton *et al.*, 2015) and jockeys (Waldron-Lynch *et al.*, 2010). Alternatively, there are some athletes that have relatively high BMDs yet still suffer from a high incidence of stress fractures, such as triathletes (McClanahan *et al.*, 2002; Maïmoun *et al.*, 2004b), rowers (Cohen *et al.*, 1995; Jürimäe *et al.*, 2006), sprinters (Maïmoun *et al.*, 2004a), jumpers (Heinonen *et al.*, 2001), distance runners (Stewart and Hannon, 2000; Maïmoun *et al.*, 2004a) and gymnasts (Kirchner *et al.*, 1995).

The implications for athletes are clear given that a stress fracture results in an average of 169 days (with a range of 90 to 270 days) of missed training (Matheson *et al.*, 1987; Ranson *et al.*, 2010). Between March 2015 and September 2016, 21 elite British triathletes suffered from 25 over-use injuries, 7 of which were medial tibial stress syndrome, that often leads to a stress response if left untreated (Detmer, 1986; Fredericson *et al.*, 1995; Anderson *et al.*, 1997; Beck, 1998; Galbraith *et*

al., 2009). A number of highly successful athletes have suffered multiple stress fractures, such as Mo Farah (2 x 5,000 m and 10,000 m Olympic Champion), Paula Radcliffe (Marathon World Record holder), Jonathan Brownlee (Triathlon Olympic silver and bronze medallist) and Alistair Brownlee (2 x Triathlon Olympic Champion). A number of studies have investigated the prevalence of stress fracture injuries in certain groups of athletes, such as track and field athletes and middle and long distance runners and have showed that the prevalence of stress fracture injury is high in these athletic groups (Hulkko and Orava 1987; Barrow and Saha, 1988; Zernicke *et al.*, 1993; Johnson *et al.*, 1994; Bennell *et al.*, 1995; Bennell *et al.*, 1996b; Bennell *et al.*, 1998; Billat *et al.*, 2001; Jones *et al.*, 2002; Iwamoto *et al.*, 2011; Tenforde *et al.*, 2013; Yagi *et al.*, 2013). Anecdotal reports suggest that elite triathletes suffer from a large number of stress fracture injuries, however, there are limited studies that have investigated stress fracture injury prevalence in elite triathletes. There are also limited studies that have explored possible causes of stress fracture injury in these athletes or possible interventions to reduce the prevalence of these injuries (Gosling *et al.*, 2008).

Bone health and stress fracture risk is influenced by non-modifiable and modifiable factors. Non-modifiable factors include; genetics, ethnicity, age, disease and sex, and modifiable factors include; mechanical loading (intensity, duration, mode, biomechanics), endocrine status, hormones and nutrition (Cashman, 2007; Vasikaran *et al.* 2011a). Further risk factors identified for stress fracture development, particularly in athletes include; increased, decreased or uncoupled bone remodelling (Parfitt, 1982; Schaffler *et al.*, 1990; Bennell *et al.*, 1996a; Burr, 2002; Schaffler, 2003), low BMD and bone thickness (Bennell *et al.*, 1999; Ackerman *et al.*, 2012; Gomez-Bruton *et al.*, 2015; Tenforde *et al.*, 2015), low calcium and vitamin D intake (Loud *et al.*, 2007; McCabe *et al.*, 2012), energy restriction (Ihle and Loucks 2004), menstrual irregularity (Ackerman *et al.*, 2012) and high running volumes (Bennell *et al.*, 1999; Tenforde *et al.*, 2015). As bone is a nutritionally modulated tissue, an individual's nutritional status is an important factor that influences both long-term bone health and acute bone turnover (Babraj *et al.*, 2005; Walsh and Henriksen, 2010), and can consequently influence stress fracture injury development. Additionally, as nutrient ingestion provides the substrates for bone collagen synthesis, but also causes the release of gastrointestinal

hormones that may play a part in the regulation of bone turnover, adequate energy availability and good dietary practices are critical for bone health in athletes.

Consuming a mixed nutrient meal suppresses all markers of bone turnover at rest (Clowes *et al.*, 2002a) and consuming individual nutrients separately (glucose, fat, protein and calcium), also suppresses bone resorption at rest (Blumsohn *et al.*, 1994a; Bjarnson *et al.*, 2002; Clowes *et al.*, 2003; Henriksen *et al.*, 2003). Guillemant *et al.* (2004) showed that calcium ingestion before and during cycling prevented the increase in carboxyterminal cross-linking telopeptide of type 1 collagen (β -CTX) concentrations and the increase in parathyroid hormone (PTH) was partially suppressed compared to the placebo trial. Barry *et al.* (2011) hypothesised that the disruption of calcium homeostasis is a potential mediator of bone loss, which is mediated by PTH. PTH secretory activity is regulated by the presence of ionised calcium (Ca^{2+}) in serum at rest, but the hypothesis that decreased Ca^{2+} triggers increased PTH during exercise has not yet been proven (Barry and Khort, 2007; Barry *et al.*, 2011). PO_4 and Vitamin D also contribute to PTH regulation, as 1,25 dihydroxyvitamin D ($1,25(\text{OH})_2\text{D}_3$) regulates the active transport of calcium and PO_4 absorption in the small intestine (Heaney and Barger-Lux, 1985; Heaney *et al.*, 2003; Martin *et al.*, 2005; Cashman, 2007). Vitamin D concentrations vary during different seasons and deficiencies are often present in winter months in athletes (Close *et al.*, 2013).

Nutrient ingestion before and during exercise has also been investigated due to the increase in bone resorption observed after prolonged and intense bouts of exercise (Guillemant *et al.*, 2004; Maimoun *et al.*, 2006; Herrmann *et al.*, 2007; Kersch-Schindl *et al.*, 2009; Scott *et al.*, 2011; Scott *et al.*, 2012), with an imbalance in bone turnover (increased bone resorption without concomitant increases in bone formation) being shown for up to 4 days after a bout of exhaustive running (Scott *et al.*, 2010). Pre-exercise and during exercise feeding showed small, transient effects on bone metabolism (Scott *et al.*, 2012; Sale *et al.*, 2015). Nevertheless, these dietary practices may not be practical for athletes as they often cause gastrointestinal distress during training sessions that may limit performance (Pfeiffer *et al.*, 2012), meaning that other nutritional interventions need to be investigated.

1.1 Fast Practitioner and Slow Researcher Model

This programme of work has an applied focus and an aim of the thesis is to produce research that can have direct impact on elite athletes. Coutts (2016) highlights the important role that research plays in the development of effective evidence-based practices in elite sport. It is common for researchers to perform laboratory-based experiments with the aim of translating the findings for practitioners. This thesis will also employ a reverse approach; working directly with elite athletes in their training environment to understand what is influencing bone metabolism and bone health. This approach will allow possible issues and areas for intervention to be identified. These areas and issues can be subsequently investigated in a laboratory setting, whilst being mindful of real life athlete practice, to ensure that the findings are applicable to elite athletes (Figure 1).



Figure1. A conceptual model for the complementary relationship between practitioners and researchers in elite sport. Redrawn from Coutts (2016).

AIMS AND OBJECTIVES

1.2 Aims

The aims of this thesis are:

1. To identify what is influencing bone metabolism in elite triathletes (Chapters 4 and 5).
2. To identify possible areas for intervention amongst elite triathletes that may be used to improve bone health and reduce the risk of stress fracture injury in this athletic population (Chapters 4 and 5).
3. To produce research that is applicable to elite athletes and has the potential to directly influence athlete behaviour in relation to bone health (Chapters 4, 5 and 7).

1.3 Objectives

The aims of the thesis will be achieved via these objectives:

1. To investigate resting bone turnover in a group of elite British triathletes (Chapter 4).
2. To record historic (retrospective) stress fracture injury occurrence in a group of elite British triathletes (Chapter 4).
3. To investigate energy intakes, macronutrient composition of the diet and calcium intakes in a group of elite British triathletes (Chapter 4).
4. To investigate dermal calcium losses during different training sessions in a group of elite British triathletes (Chapter 4).
5. To measure the amount of energy expended by elite British triathletes during 10 days of training (Chapter 4).
6. To investigate resting bone turnover, energy intakes, macronutrient composition of the diet, calcium intakes and dermal calcium losses, at a different phase of the season, in a subset of elite British triathletes and to compare this to the results obtained in the study reported in Chapter 4 (Chapter 5).
7. To investigate the dynamic nature of PTH and calcium regulation during exercise and recovery (Chapter 6).
8. To investigate the effect of feeding carbohydrate (CHO) and protein (PRO) on bone turnover, after an exhaustive run (Chapter 7).

CHAPTER 2: REVIEW OF LITERATURE

2.1 Introduction to the review of literature

This literature review will cover bone mass in endurance athletes, as it has been reported that some endurance athletes have low BMDs (Maïmoun *et al.*, 2004b; Rector *et al.*, 2008; Medelli *et al.*, 2009a; Scofield and Hecht, 2012) and some have high BMDs (Stewart and Hannon, 2000; McClanahan *et al.*, 2002; Maïmoun *et al.*, 2004a; Maïmoun *et al.*, 2004b) yet still suffer from bone injuries such as stress fractures. This literature review will examine the risk and epidemiology of stress fracture injuries in endurance athletes, as an integral part of this thesis was to provide insight into bone metabolism and bone injury in triathletes. Triathletes were studied in this thesis and were a prime focus due to the high prevalence of stress fractures amongst current elite British triathletes. Some of this information is from anecdotal reports by British Triathlon coaches and sports science support staff, that was discerned through work at the English Institute of Sport and British Triathlon as a Performance Nutritionist. Therefore, further sections of this literature review will cover the sport of triathlon and the published data surrounding bone health and bone metabolism in triathletes.

This literature review will cover the female athlete triad (the triad) and the relative energy deficit in sport (RED-S) paradigm, as almost one third of the elite triathletes studied in this thesis were female. The RED-S paradigm also highlights that a relative energy deficiency will affect many physiological aspects, not just the three entities emphasised by the triad, and it has been suggested that this phenomenon is prevalent in male athletes also (Mountjoy *et al.*, 2014). The energy deficiency caused by an imbalance between energy intake and energy expenditure is the underlying cause of both the triad and RED-S, therefore methods of measuring energy expenditures will also be examined in this literature review.

Latter sections of this literature review will cover bone remodelling, bone turnover and how bone turnover is measured, as the studies in this thesis measured bone turnover markers that provide estimations of whole body bone resorption and formation. Certain bone turnover markers that were measured in this thesis, or that are the most regularly measured markers in recent publications, will be reviewed in more detail. The literature review will go on to examine the effects of exercise (acute bouts, repeated bouts and over an athletic season) on these bone turnover markers. This is important

as the experimental studies in this thesis measured bone turnover markers after acute bouts of exercise, on consecutive mornings after numerous training sessions and during different phases of a triathlon season. Final sections will review PTH regulation and its response to exercise, as studies in this thesis investigated the PTH response to exercise, as well as calcium intakes and dermal losses. Lastly, the effect of nutrient ingestion on bone turnover markers and the relationship with exercise will also be reviewed, as these were also important aspects of this thesis.

2.2 Bone mass

Bone mass is gained and lost throughout an individual's lifetime, due to changes in the relative amounts of bone formation and bone resorption (bone turnover) at different stages of life. Skeletal growth and bone acquisition is rapid during childhood and puberty, peaking during early adulthood and then decreasing in later life, particularly after the menopause in females (Heaney *et al.*, 2000; Weaver, 2000; Bachrach, 2001; Cashman, 2007). Bone mass and structure can be assessed by techniques such as, DXA, QCT, high resolution peripheral quantitative computed tomography, magnetic resonance imaging (MRI) and finite element analysis (Banfi *et al.*, 2010).

DXA is now the most widely used and available bone densitometry technique (Blake and Fogelman, 2007; Adams, 2013). It is also considered the 'gold standard' measurement used to diagnose osteoporosis (Compston, 2005; Blake and Fogelman, 2007). DXA measurements reflect the mass of the bone but are not very sensitive to subtle changes in bone density or bone mineral content (BMC) and do not detect 3D geometry, microarchitecture or the intrinsic properties of bone matrix. In longitudinal studies it is suggested that 18 – 24 months is necessary between measures to show a significant change, unless large changes in BMD are anticipated (Gluer, 1999), for example, when using medications such as glucocorticoids or bisphosphonates, which are known to significantly change BMD. DXA scans measure BMC, bone area, areal BMD and volumetric BMD and these have been shown to be stable after reaching peak bone mass in early adulthood, until the onset of bone loss in later life (Clarke and Kholsa, 2010). There may be slight changes in these measurements

outside of the expected changes that occur with normal aging, particularly in the athletic population, due to bone adaptation to new physiological strains or loading (Ferretti *et al.*, 1998; Bachrach, 2001).

DXA measurements have also been used to determine the risk of fracture in the general population; the lower the BMD, the higher the risk of suffering a fracture, which is particularly true in terms of osteoporotic fractures (Hui *et al.*, 1989; Fujiwara *et al.*, 2003; Johnell *et al.*, 2005; Stewart *et al.*, 2006). BMD and other structural geometry measurements derived from DXA scans have previously been shown to be related to the development of stress fractures in Marine Corps (Beck *et al.*, 1996). The relative risk of fracture is increased in athletic populations, which will be discussed in subsequent sections.

2.2.1 Bone mass and mechanical loading

Mechanotransduction is the physical, biological and chemical basis of how mechanical signals (skeletal loading) are transformed into anabolic signals for bone adaptation, that is fulfilled by bone metabolism and remodelling, leading to increases in bone quantity (mass) and bone quality (Rubin *et al.*, 2006). Mechanical loading experienced by the skeletal system, caused by muscular contractions or by weight-bearing exercise, causes strain in bone tissues and matrix deformation. Bone cells experience interstitial fluid flow and dynamic pressure changes (Piekarski and Munro, 1977), pressure in the medullary cavity (Qin *et al.*, 2003) and shear forces through canaliculi (Han *et al.*, 2004). Bone remodelling is sensitive to strain magnitude (Rubin and Lanyon, 1985), the number of loading cycles (Rubin and Lanyon, 1984), the distribution of loading (Lanyon *et al.*, 1982) and the rate of strain (O'Connor *et al.*, 1982). This is also known as the 'Mechanostat Theory', which describes the mechanism of how the skeleton responds to frequently experienced strains via bone remodelling (Frost, 1987). However, the loading experienced must be dynamic, as the skeleton does not respond to static loads (Lanyon and Rubin, 1984) and the anabolic potential of the mechanical loading increases as rest periods are introduced between loading cycles (Srinivasan *et al.*, 2002). This is reflected in the lower BMDs of swimmers and cyclists compared to runners or games players. However, too much mechanical loading can cause matrix microdamage and death of nearby cells (Verborgt *et al.*, 2000).

How this mechanical loading transfers to changes in BMD or bone structure is not fully understood; the exact loads, strain magnitudes, number of loading cycles, rest periods between loading cycles or the strain rates necessary to cause positive adaptation to the bone *in vivo* are unknown. However, we do know that there are several bone cells that are sensitive to mechanical signals, including; stromal cells, osteoblasts and osteocytes, however it is difficult to designate a critically responsive cell because they are all important (Rubin *et al.*, 2006). There are also several components of the cells that could act as the mechanoreceptor, transducing a physical load into a cellular response. For example; ion channel activity in osteoblasts, stimulated by strain to the membrane (Duncan *et al.*, 1992) or by PTH (Ferrier *et al.*, 1986), activates bone cells and bone remodelling, via intracellular signalling cascades including β -Catenin, MTORC2, cAMP and intracellular calcium. With the multiplicity of mechanical signals presented to the cell, it is likely that no one mechanosensor or receptor mechanism is responsible for perceiving or responding to the mechanical loading; multiple mechanosensors are likely to interact and integrate mechanical and chemical information from the environment.

Bennell *et al.* (1997) investigated bone mass and bone turnover in a 12 month longitudinal study amongst track and field athletes and controls; DXA scans were performed at baseline and 12 months later. The results showed that differences in bone mass in different types of athletes were greatest at the sites subjected to mechanical loading, providing evidence for an osteogenic effect of mechanical loading. The greatest BMD at the lumbar spine in power athletes suggests that strain magnitude may be a more potent stimulus than strain frequency. The hypothesis that high-magnitude strains applied at a high rate with relatively few strain cycles, produces maximal osteogenesis, has been proven in animal models of controlled external loading (O'Connor *et al.*, 1982; Rubin and Lanyon, 1984; Rubin and Lanyon, 1985; Raab-Cullen *et al.*, 1994). If this theory is applied to humans, sports such as weight lifting, sprinting and jumping should have a greater osteogenic effect than distance running, which is characterised by lower-magnitude strains with repetitive strain cycles. Bennell *et al.* (1997) showed that the endurance runners had a greater bone mass than controls at lower limb sites,

suggesting that the ground reaction forces applied to the lower limbs during running training provide an osteogenic response to the bones of the lower limbs only.

This site-specific osteogenic response may be due to the propagation of ground reaction forces from the foot upwards (Light *et al.*, 1980; Snow-Harter *et al.*, 1992), meaning that a 'strain threshold' is not reached at sites further away from where the ground reaction force was applied. This explains why Bennell *et al.* (1997) showed the least difference in BMD between endurance athletes and controls at proximal sites such as the femur and lumbar spine. Lohman *et al.* (1995) and Bennell *et al.* (1997) proposed that there may be a local effect of mechanical loading on net bone formation and that exercise may influence the pattern in which BMD is distributed throughout the skeleton without necessarily increasing the total amount of BMC.

Jürimäe *et al.* (2006) showed that arm BMD increased significantly after 6 months of intense training, in 12 well-trained rowers, but whole body BMD and BMC remained unchanged. Arm BMD in the rowers is similar to leg BMD in the runners (Bennell *et al.*, 1997), which supports the importance of impact activity on bone development. The authors suggest that the bone adaptation threshold at the arms may be lower than at other sites, due to the bones of the arms not being exposed to as much mechanical loading as the bones of the lower limbs or spine, due to walking and other day to day activities. Therefore when the bones of the arms are weight trained the adaptation threshold is likely to be surpassed (Lohmann *et al.*, 1995; Hsieh *et al.*, 2001).

Bennell *et al.* (1997) showed that lower limb BMD did not change in any group, which, was attributed to the athletes maintaining baseline levels of training throughout the 12 month study, making it plausible that bones of the lower limbs had already adapted to this amount of mechanical loading. Achieving additional gains in bone mass may require an increase in training load or intensity, in order to reach the new elevated threshold for osteogenic bone adaptation. The osteogenic bone adaptation threshold has not yet been quantified in humans, but Brahm *et al.* (1997a) established that running for approximately 7 hours per week has a positive effect on bone metabolism and suggests that there may be a threshold of around 80 – 100 km per week, around which the effects of running

are positive, but above this and the effects are negative (Frost, 1987; Burrows *et al.*, 2003). An increase in training load may occur at different points of an athlete's season or lifetime; for example, the training load will change significantly during an Olympic cycle, meaning that sometimes it may exceed the bone adaptation threshold, and other times not. When a junior athlete transitions to senior level, which will coincide with a significant increase in training load, this is likely to surpass the adaptation threshold. Anecdotal reports by British Triathlon coaches, suggest that this increase in training load does not always remain osteogenic and often causes over-use and stress fracture injuries in many cases. Studies have reported that stress fracture injury occurrence increases following a sudden change in training routine, such as a change from low volumes to high volumes of training (Goldberg and Pecora, 1994; Duckham *et al.*, 2014).

2.2.2 Bone mass measurements in athletes

Hetland *et al.* (1993) showed that lumbar BMC was 19% lower in elite runners (that cover ≥ 100 km per week) than non-runners. In addition the authors showed that the elite runners had increased bone turnover by 20 – 30% compared to the controls. However this study provides a cross-sectional view during only one phase of training and there is a lack of longitudinal research that has investigated the change in both bone turnover and bone mass in endurance athletes over the course of a season or number of seasons. There are however some studies that have investigated seasonal variation in BMD in athletes such as triathletes and basketball players.

Klesges *et al.* (1996) showed a 6.1% decrease in total BMC and a 10.5% decrease in the bones of the lower limbs, over 10 months in collegiate basketball players. The authors attributed the decreased BMC measurements to dermal calcium losses, and in accordance showed that calcium supplementation was associated with increased BMC. McClanahan *et al.* (2002) measured BMD in 21 amateur triathletes and showed that there was no significant difference in whole body BMD over 6 months of training (1.082 ± 0.091 vs 1.083 ± 0.091 g.cm⁻²). Calcium intake was recorded at week 12 and week 24, however only the average of the two time points was reported as 994 ± 519 mg.day⁻¹. Overall dietary intake and training loads were not reported so it is not known whether these

changed significantly throughout the 6 month period, despite their potential influence on BMD (Specker, 1996; New *et al.*, 1997; Nattiv *et al.*, 2007; Mountjoy *et al.*, 2014).

Bennell *et al.* (1997) measured BMC and BMD at baseline and 12 months later in 50 power athletes, 61 endurance athletes and 55 nonathlete controls and bone turnover markers (osteocalcin; OC and urinary Pridinoline; u-PYD) were measured at baseline. Compared with controls, male power athletes had higher BMDs at all regional sites, whereas male endurance athletes had higher BMD at lower limb sites only. The annual change in total body BMC ranged from 1.6 – 1.8% in all male groups. Lumbar spine BMD increased the most in power athletes and tibia/fibula BMD did not change in any group within 12 months. Neither of the bone turnover markers predicted 12 month changes in bone mass, however a single measurement of two bone turnover markers is an inadequate measure of bone turnover, which can respond acutely to various external factors.

Stewart and Hannon (2000) tested 12 competitive runners, 14 cyclists, 13 athletes that performed both running and cycling ('both' group) and 23 nonexercising controls. The authors showed that the runners had the highest total BMD, leg BMD and leg BMC out of the 4 groups. Only the 'both' group had greater spine BMD than the runners and the cyclists had lower arm BMD than the runners. As there were no anthropometric differences between any of the athletic groups, yet the BMD and BMC measurements varied amongst athletes, it is possible that the differences in measurements may be partly attributed to differences in exercise and training, particularly during childhood and adolescence (Tenforde and Fredericson, 2011; Duckham *et al.*, 2013; Gomez-Bruton *et al.*, 2015). Only one scan was performed in this study, so this data only provides a cross-sectional view of BMD. The authors explain that skeletal mass can be improved during adult life by performing impact bearing activities, which will probably be site-specific. Adaptations to BMD and bone structure throughout a season as mechanical loading changes is yet to be investigated, as repeated DXA scans (more than two) are not often performed in athletes or other healthy individuals.

A case study by Zanker *et al.* (2004) highlighted an extreme case of low BMD in a female endurance athlete. Repeated BMD measures were made over a period of 12 years. The athlete suffered from

primary amenorrhea and osteoporosis at age 24.8 years and had a weekly training distance of 90 – 100 km of running, 100 – 120 km of cycling and 5 – 6 km of swimming. The athlete had also suffered multiple bilateral stress fractures of the tibia and metatarsals. When the athlete lost 3.7 kg in body mass, BMD decreased by 9.8% at the lumbar spine and by 12.1% at the proximal femur. When the individual was treated with oestradiol skin patches and norethisterone, lumbar spine BMD increased by 9.4% and when the individual gained 8.1 kg in body mass, proximal femur BMD increased by 16.9%, but with no further increase at the lumbar spine. Body mass changes in endurance athletes may occur throughout the competitive season, with body mass losses occurring during high training loads due to increased energy expenditure and/or inadequate energy intake. Therefore, this warrants regular DXA or QCT scans throughout a competitive season, particularly if athletes display any risk factors for the female athlete triad or RED-S and this may help to maintain or improve bone health if necessary. However, a limitation of this and other case studies is that it is not possible to make recommendations for treatment or prevention of poor bone health in other athletes based on the observations of one extreme case.

2.3 Stress fracture injuries

A stress fracture can be defined as a partial or complete fracture of a bone that is a result of repeated stress applied to the bone that is less than the force required to fracture a bone in a single application (Martin and McCulloch, 1987). The development of a stress fracture involves repetitive loading, bone fatigue, microdamage accumulation and increased bone remodelling (Grimston and Zernicke, 1993; Bennell *et al.*, 1996a). Stress fracture development should be seen as a process rather than an event and is therefore the unsuccessful process of the bone adapting to a change in the mechanical environment caused by repetitive loading (Bennell *et al.*, 1996a).

The skeleton has mechanisms that prevent the progression of microdamage, that under normal circumstances function effectively to prevent stress fracture development. One of these mechanisms is the remodelling process. Bennell *et al.* (1996a) postulated two hypotheses that may explain the development of a stress fracture. The first is the Primary Microdamage Hypothesis and suggests that

microdamage occurs first due to repetitive loading and a remodelling response is initiated, in order to repair the microdamage that has accumulated (Straus, 1932, Roberts *et al.*, 1939; Schaffler *et al.*, 1989; Frost, 1991). The second is the Primary Remodelling Hypothesis and this suggests that accelerated bone remodelling caused by diet, exercise and hormonal factors causes weakened sites on the bone surface (Murguia *et al.*, 1988; Mori and Burr, 1993). When subsequent bone strain occurs, microdamage may accumulate at these already weakened areas. In both cases, if microdamage accumulation exceeds repair by the remodelling process, a stress fracture may develop (Figure 2). The difference between the two hypotheses is determined by whether bone remodelling precedes or follows microdamage production. The bone remodelling process will be discussed in latter sections of this literature review, but as the process is cyclical, it may be that these two hypotheses overlap and both mechanisms occur simultaneously.

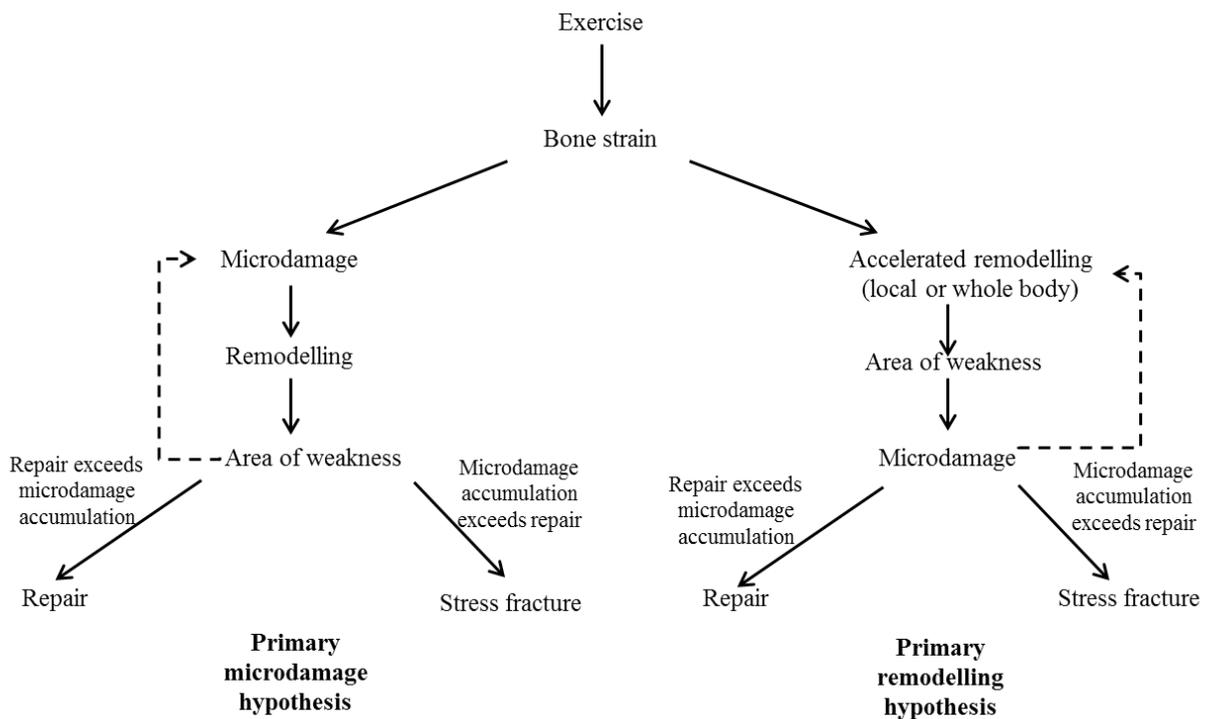


Figure 2. Redrawn from Bennell *et al.* (1996a). Possible mechanisms for stress fracture development; the Primary Microdamage Hypothesis and the Primary Remodelling Hypothesis.

A stress fracture injury is a debilitating injury for an athlete and depending on the severity of the injury can result in an average of 169 days (range of 90 to 270) of restricted weight-bearing activity and therefore missed training (Matheson *et al.*, 1987; Ranson *et al.*, 2010). As previous stress fracture is a risk factor for future stress fractures (Nattiv *et al.*, 2000; Kelsey *et al.*, 2007), it is vital that more research is conducted into how stress fracture injury can be prevented in the first instance, to minimise the chance of recurrent stress injuries and therefore large amounts of missed training time.

2.3.1 Risk factors for the development of stress fracture injury

Some of the risk factors identified for stress fracture development, particularly in athletes include; increased bone remodelling and bone turnover, low BMD and bone thickness, low calcium and vitamin D intake, energy restriction, menstrual irregularity, high running volumes and reduced lower leg muscle strength (Bennell *et al.*, 1999). Figure 3 summarises the contribution of risk factors to stress fracture pathogenesis.

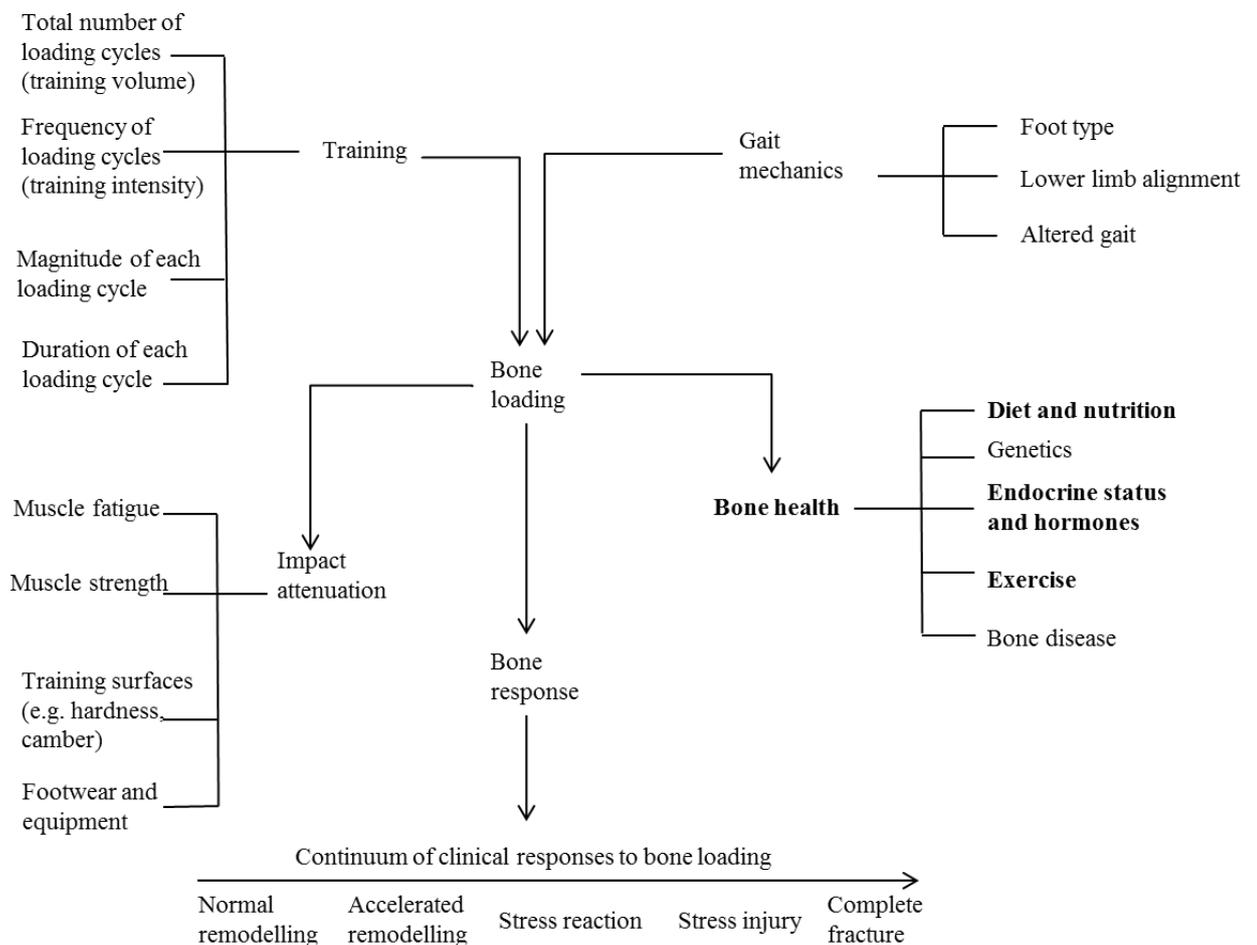


Figure 3. Redrawn from Bennell *et al.* (1999). A summary of the contribution of risk factors to stress fracture pathogenesis. Bone health is a major factor that determines how the bone responds to loading. Factors that affect bone health such as, diet and nutrition, endocrine status and exercise were key components of this thesis.

Accelerated bone remodelling and bone turnover, which can result from excessive bone strain or other systemic factors (Bennell *et al.*, 1999), can predispose the bone to stress fracture development due to increased microdamage accumulation and therefore weakening of the bone at specific sites. Bennell *et al.* (1998) studied 95 track and field athletes that developed 20 stress fractures over the 12 month observation period. OC, PYD, deoxypyridinoline (DPD), and aminoterminal cross-linking telopeptide of type 1 collagen (NTX) were measured once a month to reflect bone formation and resorption. There was no difference in bone turnover marker concentrations between those that obtained a stress fracture injury and those that did not, suggesting that biochemical markers of bone turnover may not be the best predictors of stress fracture injury in athletes. However, there could have been changes in bone turnover that occurred between the monthly samples and due to the responsiveness and sensitivity of bone turnover to various external factors (which will be discussed in later sections), the monthly measurements may not have been frequent enough to detect changes in bone turnover.

Stress fractures are more likely to develop in the bones of the lower limbs, such as the tibia, fibula, metatarsals, navicular, femur and pelvis (Hulkko and Orava, 1987; Bennell *et al.*, 1996b), due to the greater loads applied to these extremities through ground reaction forces (Light *et al.*, 1980; Snow-Harter *et al.*, 1992). The lower limbs are often subject to exercise that consist of repetitive loading, for example, running produces ground reaction forces nearly five times greater than walking (Wasserstein and Spindler, 2015), but is usually in a single plane and at a constant rate with little variation during long distance races or training runs (Rubin and Lanyon, 1984; Burr *et al.*, 1985; Burr, 2002; Robling, 2009). A further risk factor amongst athletes, particularly in those that have high running volumes, is reduced lower leg muscle strength. The muscles may protect the tibia by counteracting the joint and ground reaction forces applied to the bone, so if the muscles are weakened

or fatigued due to injury or overtraining, the tibia may be less protected (Martin and McCulloch, 1987; Bergman *et al.*, 2004).

Loud *et al.* (2007) showed that female athletes that had suffered a stress fracture had lower spine BMD compared to controls, after controlling for menstrual function and physical activity. Similarly, Bennell *et al.* (1996b) showed that female track and field athletes with lower BMD at the spine were at a greater risk of developing a stress fracture. However, as stress fractures are more likely to develop in the lower limbs (Hulkko and Orava, 1987; Bennell *et al.*, 1996b), the relevance of low BMD at the spine is questionable. Another study in female athletes showed that after adjusting for body weight, those that had previously suffered from stress fractures, had thinner tibial cross-sectional area, lower BMD and less cortical area in the posterior tibia (Schnackenburg *et al.*, 2011). As bone strength is also related to bone structure and geometry (Nordin and Frankel, 1989), specific measures of these, such as cross-sectional area or cortical thickness, at likely fracture sites such as the tibia, are more applicable measures of stress fracture risk (Giladi *et al.*, 1987; Milgrom *et al.*, 1988; Milgrom *et al.*, 1989; Beck *et al.*, 1996; Crossley *et al.*, 1999). There are several studies that have investigated the relationship between BMD, bone structure and stress fracture risk in athletes (Carbon *et al.*, 1990; Myburgh *et al.*, 1990; Grimston *et al.*, 1991; Crossley *et al.*, 1999; Ackerman *et al.*, 2012; Gomez-Bruton *et al.*, 2015; Tenforde *et al.*, 2015).

Calcium and vitamin D intake is also related to stress fracture injury (Medelli *et al.*, 2009a; Nieves *et al.*, 2010; Tenforde *et al.*, 2010; Sonnevile *et al.*, 2012; Wentz *et al.*, 2012). A review paper (McCabe *et al.*, 2012) identified 3 studies that showed positive correlations between vitamin D intake and protection against stress fracture injury (Givon *et al.*, 2000; Ruohola *et al.*, 2006; Lappe *et al.*, 2008), while 2 studies showed no association (Loud *et al.*, 2005; Välimäki *et al.*, 2005). Studies such as that by Loud *et al.* (2005) are limited by the retrospective survey-based method of recalling calcium and vitamin D intakes. Nieves *et al.* (2010) showed that higher intakes of calcium, milk and servings of dairy products per day, were each related to reduced rate of stress fracture in 125 female cross country runners, where the incidence was 14.4% over 2 years. In a randomised, double-blind, placebo-controlled trial, Lappe *et al.* (2008) assessed the incidence of stress fracture injuries over 8

week training periods in female Navy recruits, which ran between May 2001 and March 2006. The recruits were randomised to 2000 mg of calcium and 800 IU vitamin D per day or a placebo. The authors showed that out of the 3,700 recruits, 309 were diagnosed with a stress fracture, with a 21% lower incidence in the supplemented group compared to the placebo group. This highlights the importance of calcium and vitamin D intakes and the need for supplementation if deficiencies are present, which are common in athletes (Close *et al.*, 2013). However, Lappe *et al.* (2008) did not account for differences in sun light exposure between recruits across the 5 years that the study was performed, which may have varied throughout the study period (Webb *et al.*, 1988).

Inadequate energy intakes can increase the risk of stress fractures, particularly if coupled with abnormal or restrictive eating habits. Chronic energy deficit, due to restricted energy intake or excessive energy expenditure, causes weight loss and compensatory suppression of endocrine function and other physiological systems, including reproductive function (De Souza and Williams, 2005; Manore *et al.*, 2007; Melin *et al.*, 2014). Suppressed oestrogen release in females is associated with stress fractures, decreased BMD, failure to attain peak bone mass in early adulthood, and brittle bone diseases in later life such as, osteoporosis and osteopenia (De Souza and Williams, 2005; De Souza *et al.*, 2008). Similarly, suppressed reproductive hormones involved in the hypothalamic-pituitary-gonadal axis, such as testosterone in males, causes hypogonadotropic hypogonadism, which predisposes male athletes to bone stress injuries (Bennell *et al.*, 1996c; Tenforde *et al.*, 2016). Ihle and Loucks (2004) showed that reduced energy availabilities, to $30 \text{ kcal}\cdot\text{kgLBM}^{-1}\cdot\text{d}^{-1}$, cause the suppression of bone formation, and also cause a wide range of metabolic hormones to be affected such as, 3,3,5-triiodothyronine (T_3) and insulin growth factor-1 (IGF-1).

Late menarche appears to be a risk factor for stress fracture development in female athletes and this is probably caused by other factors, such as, excessive training, low energy intakes and low body weight (Bennell *et al.*, 1995; Duckham *et al.*, 2012; Tenforde *et al.*, 2015; Mallinson *et al.*, 2016). An abnormal menstrual cycle is also a risk factor for stress fracture development; oestrogen increases bone mass by dampening osteoclastic bone resorption (Lindsay *et al.*, 1976; Christiansen *et al.*, 1981; Kameda *et al.*, 1997), and those with amenorrhea or oligomenorrhea have disrupted oestrogen release

as well as disrupted luteinising hormone and follicle-stimulating hormone pulsatility (Fisher *et al.*, 1986; Grinspoon *et al.*, 1999). Several studies have demonstrated that amenorrheic or oligomenorrheic athletes have an increased risk of stress fracture injury, as well as developing osteoporosis in later life (Clark *et al.*, 1988; Myburgh *et al.*, 1990; Winfield *et al.*, 1997; Bennell *et al.*, 1999; Grinspoon *et al.*, 1999; Rauh *et al.*, 2006; Shaffer *et al.*, 2006). Amenorrhea is one of the three entities of the female athlete triad, along with low energy availability and poor bone health (Nattiv *et al.*, 1994). More recently, the RED-S concept has been proposed due to the recognition that these factors are not exclusive to females. RED-S also refers to impaired physiological function including, but not limited to, metabolic, reproductive, bone, immunological, gastrointestinal and cardiovascular health (Mountjoy *et al.*, 2014), which will be discussed in subsequent sections.

Despite the recent recognition of these entities in male athletes the majority of studies have investigated this in female athletes only (Tenforde *et al.*, 2016) and there is a lack of evidence for RED-S occurring in male athletes. Hetland *et al.* (1993), Stewart and Hannon (2000) and Hind *et al.* (2006) have showed that male endurance runners suffer from low BMD, and Vogt *et al.* (2005) and Müller *et al.* (2006) have reported that male cyclists and ski jumpers have severely reduced energy availabilities, but these factors have not been linked to stress fracture injury in males. Several studies have explored reduced reproductive function (hypogonadotropic-hypogonadism) in male endurance athletes (Mathur *et al.*, 1986; McColl *et al.*, 1989; Hackney *et al.*, 1988; Hackney *et al.*, 1990; Wheeler *et al.*, 1991; Roberts *et al.*, 1993; De Souza *et al.*, 1994; Hackney *et al.*, 1998; Skarda and Burge, 1998) but there are less studies that investigate this in relation to bone health and stress fracture risk (Smith and Rutherford, 1993; Bennell *et al.*, 1996c; Maïmoun *et al.*, 2003; Vinther *et al.*, 2008).

2.3.2 Stress fractures in athletes

During a 14 year period, Hulkko and Orava, (1987) treated 368 stress fractures in 324 athletes, 72% of which occurred in runners. International level runners experienced the greatest risk of multiple fractures and poor bone healing. In a study of 230 runners over three years, 21 (9.1%) of the athletes suffered a stress fracture (Yagi *et al.*, 2013). Similarly, a study of 701 cross country runners identified

a 5.4% and 4% rate of stress fractures in female and male adolescents across 2 – 3 seasons (Tenforde *et al.*, 2013). Billat *et al.* (2001) recorded that top-class marathon runners cover over 200 km per week and run twice daily, therefore it is not surprising that the highest incidence rates of stress fractures occur in these athletes (Johnson *et al.*, 1994; Bennell *et al.*, 1996b; Jones *et al.*, 2002; Iwamoto *et al.*, 2011). A series of studies undertaken by Bennell and colleagues mapped stress fracture epidemiology particularly across track and field athletes. Bennell *et al.* (1995) showed that in 53 female athletes there were 45 incidences of stress fracture injuries throughout the participants' lifetimes. Bennell *et al.* (1996b) showed that over 12 months, 26 stress fractures were reported in 111 track and field athletes, resulting in an incidence rate of 21.1%. Bennell *et al.* (1998) showed that over 12 months, 20 stress fractures were obtained in 95 track and field athletes. It should be noted that ascertainment or publication bias may be present in these studies that have used questionnaires to retrospectively assess stress fracture injury incidence, due to athletes that are concerned about the topic, *i.e.* if they have previously suffered from a stress fracture, being more likely to respond to questionnaires and participate in the research.

Although the epidemiology and risk of stress fracture injury has been fairly well documented in runners and track and field athletes, there are limited studies that have investigated this risk amongst triathletes. Triathlon is a sport where anecdotally, athletes suffer from a large number of stress fractures and overuse injuries, but there are only a few studies that have recorded this, and there are no studies that have investigated this in elite Olympic triathletes. This high prevalence is probably due to the high training volumes and having three disciplines to practice, two of which are non-weight-bearing (swimming and cycling), followed by a weight-bearing run, which also means that triathletes have little recovery time between sessions. Andersen *et al.* (2013) showed that the average prevalence of overuse injuries was 56% amongst 174 amateur triathletes participating in a long distance triathlon; further research is therefore warranted in this area.

Prevention studies in military recruits and athletes have investigated the use of shock absorbing orthotics in marching boots, along with a reduction in the frequency and intensity of running sessions, pre-exercise leg muscle stretching and oral calcium and vitamin D supplementation (Belkin, 1980;

Scully and Besterman, 1982; Sterling *et al.*, 1992; Ross and Woodward, 1994; Jones *et al.*, 2002; Lappe *et al.*, 2008). There is, however, a lack of studies that have investigated how stress fracture injuries can be prevented in athletes, perhaps because of the difficulty in carrying out intervention studies over prolonged periods in elite athlete populations. It is therefore important to investigate the risk of stress fracture injury and possible ways to reduce the risk in triathletes.

2.4 Triathlon

Triathlon is a sport consisting of three different events; swimming, cycling and running. There are five different triathlon races; super sprint, sprint, standard, middle distance and long distance, the distances are shown in Table 1. Triathlon is a relatively new sport; becoming an Olympic sport for the first time in Sydney 2000 and participation in triathlon has increased in recent years (Strock *et al.*, 2006; Gosling *et al.*, 2008). Olympic triathletes compete in the standard distance and there are currently 11 males and 10 females in the British Triathlon team (2015 – 2016 World Class funding).

Table 1. Triathlon race distances.

	Distance (km)		
	Swim	Bike	Run
Super sprint	0.4	10	2.5
Sprint	0.75	20	5
Standard (Olympic)	1.5	40	10
Middle distance	1.9	90	21
Long distance	3.8	180	42

There are a number of studies that have investigated triathlon training and injuries (Ireland and Micheli, 1987; Massimino *et al.*, 1988; Williams *et al.*, 1988; O'Toole *et al.*, 1989; Collins *et al.*, 1989; Migliorini *et al.*, 1991; Korkia *et al.*, 1994; Wilk *et al.*, 1995; Manninen and Kallinen, 1996; Cipriani *et al.*, 1998; Vleck and Garbutt, 1998; Fawkner *et al.*, 1999; Clements *et al.*, 1999; Burns *et al.*, 2003; Egerman *et al.*, 2003; Shaw *et al.*, 2004; Burns *et al.*, 2005; Villavicencio *et al.*, 2006;

Andersen *et al.*, 2013). A review of these studies concluded that there is a significant lack of published information describing injuries, the profile of injuries sustained and evidence for the prevention of injuries in triathlon (Gosling *et al.*, 2008). These studies are limited by retrospective designs or inconsistencies in the definitions used to define injuries, further, training regimes are complex and varied, meaning it is difficult to measure risk exposure to injury.

There are 8 studies that have investigated training and injuries in ‘elite’ triathletes (Massimino *et al.*, 1988; O’Toole *et al.*, 1989; Collins *et al.*, 1989; Migliorini *et al.*, 1991; Vleck and Garbutt, 1998; Clements *et al.*, 1999; Egerman *et al.*, 2003; Villavicencio *et al.*, 2006). Only one of these studies investigated injury and training characteristics of elite Olympic triathletes (Vleck and Garbutt, 1998). Vleck and Garbutt (1998) investigated overuse injuries in 12 elite, 17 development and 87 club level male triathletes, using a 5 year retrospective questionnaire. Overuse injury occurred in 75% of elite and development triathletes, with 2 – 3 sites being affected by injury and running accounting for more of the total number of injuries than swimming and cycling in the elite triathletes. This study is limited by the retrospective design and the reliance on subjective recall. Scofield and Hecht (2012) highlighted that very limited research has been implemented on the topic of bone health in triathletes, but suggest that the amount of cross-training performed by triathletes may be more beneficial for bone compared to competing in a single discipline, particularly cycling or swimming alone. The authors fail to address the huge volume of training performed by triathletes in comparison to athletes of single disciplines and the subsequent effects this has on energy availability, the repetitious nature of the training and the lack of rest and recovery, all of which influence bone health. A unique aspect of a triathlon race is the transition from two non-weight-bearing activities (swimming and cycling) to a high intensity, weight-bearing run and the effect that performing all three activities in succession has on both the acute bone turnover response but also on long-term bone health is unknown.

A number of studies have investigated nutritional practices of elite triathletes, but used self-reported diet diaries as the main outcome measure (Burke and Read, 1987; Burke *et al.*, 1991; Frentsos and Baer, 1997; Bentley *et al.*, 2007; Cox *et al.*, 2010). Cox *et al.* (2010) collected food and fluid diaries from 51 elite male and female Australian triathletes and showed that the triathletes consumed

adequate CHO intake pre-race. The triathletes failed to consume adequate CHO intake during the race (in relation to recommended guidelines; Burke *et al.*, 2001) and this was attributed to limited opportunities to ingest the CHO and gastrointestinal intolerance. Given the importance of nutritional status on bone health and also performance in triathlon, there are a lack of studies that have investigated bone health and nutritional practices in elite Olympic triathletes.

2.5 The Female Athlete Triad

The triad was first identified by Yeager *et al.* (1993) and Nattiv *et al.* (1994) and is the interrelationship between energy availability, menstrual function and bone health (Figure 4).

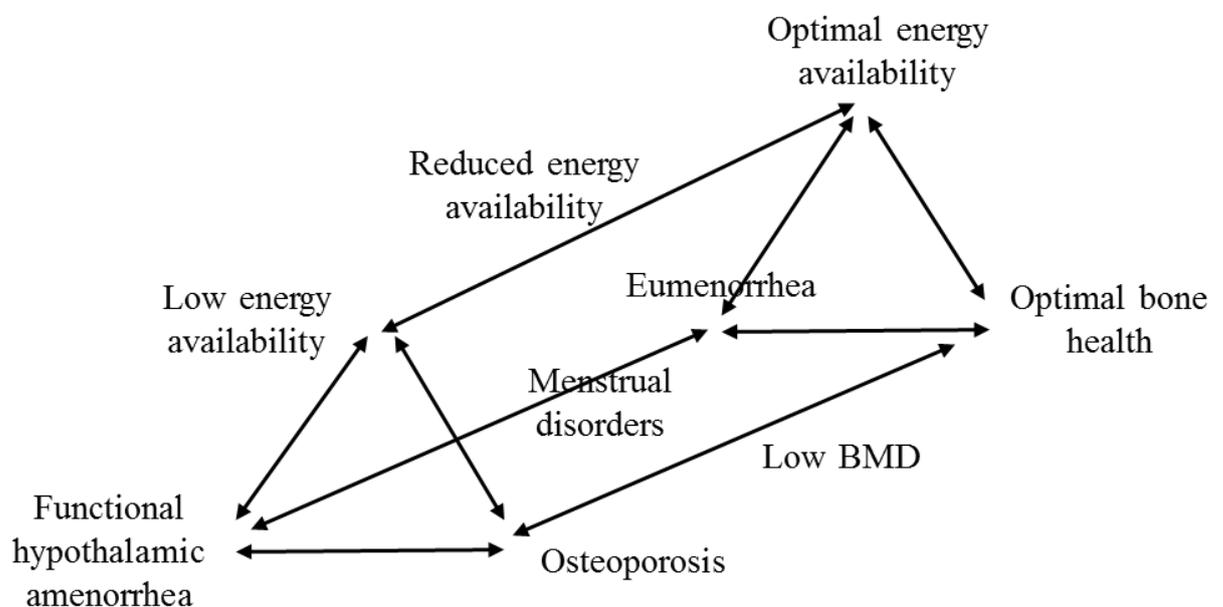


Figure 4. Redrawn from Nattiv *et al.* (2007) ACSM Position Stand The Female Athlete Triad. This figure represents the spectrums of energy availability, bone health and menstrual function that female athletes can move along.

When an athlete is suffering from the unhealthy conditions characterised by the triad, low energy availability can impair bone health by inducing amenorrhea and therefore removing the protective effect of oestrogen on osteoclastic bone resorption (Eastell, 2005). This can also suppress other hormones such as IGF-1, T3, insulin and leptin that promote bone formation (Nattiv *et al.*, 2007).

Similarly, low energy availability can alter levels of other metabolic hormones and substrates, such as, cortisol, growth hormone, ghrelin, peptide-tyrosine-tyrosine, glucose, fatty acids and ketones (Loucks and Thuma, 2003; Wade and Jones, 2004; Melin *et al.*, 2014).

Energy availability is defined as dietary intake minus exercise energy expenditure, relative to lean body mass (Nattiv *et al.*, 2007). When energy availability is low (usually at or below 30 kcal·kgLBM⁻¹·d⁻¹) physiological mechanisms reduce the amount of energy needed for physiological processes, such as cell repair, thermoregulation, immunity, growth, bone turnover and reproduction (Wade *et al.*, 1996). Luteinising hormone pulsatility and therefore reproductive function (Loucks and Thuma, 2003) and bone formation (Ihle and Loucks, 2004) are compromised abruptly at a threshold of energy availability (≤ 30 kcal·kgLBM⁻¹·d⁻¹), which corresponds closely to resting metabolic rate (Loucks *et al.*, 2011).

In athletes, low energy availabilities (< 45 kcal·kgLBM⁻¹·d⁻¹, which usually corresponds to energy balance), can occur by increased exercise energy expenditures without sufficient energy intakes or by reducing energy intakes but maintaining exercise energy expenditures. This can occur through purposeful dieting, abnormal eating practices, eating disorders, or excessive exercise, or can be inadvertent, due to the lack of a strong biological drive for athletes to match their energy expenditures with increased energy intakes (Truswell, 2001; Nattiv *et al.*, 2007). This could also be due to the appetite suppressing effect of exercise (King *et al.*, 1994; King *et al.*, 1997; Hubert *et al.*, 1998), which is more pronounced when consuming a high carbohydrate diet as endurance athletes tend to do (Horvath *et al.*, 2000a; Horvath *et al.*, 2000b; Stubbs *et al.*, 2004). Risk factors for athletes suffering low energy availabilities include; starting a sport from a young age, particularly one that favours leanness for performance, dieting to meet race weight or body composition goals, injury, and a sudden increase in training volume (Sundgot-Borgen *et al.*, 1994; Sundgot-Borgen, 2002).

Menstrual disorders result from disrupted luteinizing hormone pulsatility (Loucks *et al.*, 1989; Laughlin *et al.*, 1996), which has been shown to be disrupted at energy availabilities of 30 kcal·kgLBM⁻¹·d⁻¹ (Loucks and Thuma, 2003). The energy availability of amenorrhoeic athletes is

consistently less than $30 \text{ kcal}\cdot\text{kgLBM}^{-1}\cdot\text{d}^{-1}$ (Drinkwater *et al.*, 1984; Deuster *et al.*, 1986; Keiserauer *et al.*, 1989; Myerson *et al.*, 1991; Wilmore *et al.*, 1992; Kopp-Woodroffe *et al.*, 1999; Thong *et al.*, 2000). Menstrual disorders can be classed as oligomenorrhea, primary amenorrhea or secondary amenorrhea. Oligomenorrhea is classified as menstrual cycles occurring at intervals longer than 35 days, primary amenorrhea is classified as no menarche by the age of 15 years and secondary amenorrhea is classified as the absence of menstrual cycles lasting more than 3 months once menses have begun (Practice Committee of the American Society for Reproductive Medicine, 2004). An athlete can still have what appear to be normal menstrual cycles, but have a short luteal phase and therefore luteinizing hormone release is impaired. Alternatively, athletes can have anovulation where there is no ovulation during the menstrual cycle. Therefore, female athletes could be at risk of suffering from impaired oestrogen release, resulting in a lack of osteoclast activity suppression, without being aware of it. The prevalence of secondary amenorrhea has been reported to be as high as 65% in long distance runners (Dusek, 2001). The prevalence of amenorrhea has been shown to increase from 3% to 60% as running mileage increases from 13 to 113 km per week, which coincided with a decrease in body weight from 60 to 50 kg (Sanborn *et al.*, 1982).

The triad focuses on BMD as the measure of bone health, despite this, BMD is only one aspect of bone strength and is not the best predictor of fracture or stress fracture risk in athletes (Leib *et al.*, 2004). When measured by DXA, BMD is expressed as T-scores and Z-scores, which are used to compare individuals of the same age and sex. The World Health Organisation has defined osteoporosis in postmenopausal women as a T-score ≤ -2.5 and in the rest of the population a Z-score of ≤ -2.0 coupled with another clinical risk factor for fracture indicates osteoporosis. Clinical risk factors include; chronic undernutrition, eating disorders, menstrual disorders, hypogonadism, glucocorticoid exposure and previous fractures. A Z-score of -1.0 or below in an athlete warrants investigation, especially if coupled with another risk factor. Athletes involved in weight-bearing sports should have a 5 – 30% greater BMD than non-active individuals of the same age (Fehling *et al.*, 1995; Nichols *et al.*, 2000; Nichols *et al.*, 2007; Tenforde *et al.*, 2015), however poor nutritional status and impaired menstrual function can attenuate the beneficial effects of exercise in athletes (Pearce *et al.*, 1996; Christo *et al.*, 2008; Ackerman *et al.*, 2012).

BMD starts to decline as amenorrhea progresses, this happens gradually and the loss of BMD may not be fully reversible (Warren *et al.*, 2002). The relative risk for stress fracture injury is two to four times greater in amenorrheic than eumenorrheic athletes and increases further when nutritional deficiencies and a low BMD is present (Bennell *et al.*, 1999). Barrack *et al.* (2014) showed that the risk of a bone stress injury increased from 15 – 20% when a single risk factor was present, to 30 – 50% when two or more risk factors were present. Risk factors include BMD Z-score <-1.0, BMI <21, purposeful exercise >12 h·wk⁻¹, participation in a leanness sport, elevated dietary restraint and abnormal menstrual status.

2.6 Relative Energy Deficit in Sport (RED-S)

Recently it has become evident that a relative energy deficiency does not affect only three entities, but actually affects many aspects of physiological function (Mountjoy *et al.*, 2014), including but not limited to; metabolic rate, reproductive function, bone health, immunity, endocrine systems, growth and development, cardiovascular systems, gastrointestinal systems and psychological health. It is also more evident that this syndrome can occur in men, and therefore a new terminology was created; RED-S.

The underlying problem of RED-S is a decreased energy availability that is inadequate to support physiological functions involved in health and performance. When energy availability decreases, these functions are compromised in order to reduce the energy they require. This leads to a disruption in hormones and other metabolic characteristics (Loucks, 2004). Although the literature on low energy availability and the symptoms of RED-S focuses on female athletes (Mountjoy *et al.*, 2014), research has shown that a high number of male athletes also have severely restricted energy availabilities, low BMD and altered endocrine function (Hetland *et al.*, 1993; Stewart and Hannon, 2000; Nichols *et al.*, 2003; Ferrand and Brunet, 2004; Vogt *et al.*, 2005; Hind *et al.*, 2006; Muller *et al.*, 2006; Hackney, 2008; Rector *et al.*, 2008; Smathers *et al.*, 2009; Sundgot-Borgen and Torstveit,

2010; Guillaume *et al.*, 2012; Sundgot-Borgen *et al.*, 2013; Tenforde *et al.*, 2015), which is most common in endurance athletes.

Top level endurance athletes such as marathon runners are likely to have particularly low energy availabilities. Fudge *et al.*, (2006) demonstrated through the use of doubly labelled water (DLW), the gold standard measure of energy expenditure, that elite male Kenyan runners have energy availabilities as low as 6 kcal·kgLBM⁻¹·d⁻¹. Drenowatz *et al.* (2012) demonstrated that athletes ranging from 10 km runners to Ironman distance triathletes have energy availabilities ranging from 24 kcal·kgLBM⁻¹·d⁻¹ to 33 kcal·kgLBM⁻¹·d⁻¹.

Ihle and Loucks (2004) examined the dose-response relationship between energy availability and bone turnover in sedentary females. Generally, energy availability is balanced, *i.e.* energy expenditure is equal to energy intake at 45 kcal·kgLBM⁻¹·d⁻¹, although this may not be the case in athletes of small body size, as resting metabolic rate is underestimated in the linear scaling of energy availability relative to lean body mass (Loucks *et al.*, 2011). In the study, energy availability was reduced to 10 kcal·kgLBM⁻¹·d⁻¹, by exercise and a severe energy restriction, a value which is not far from practices of some amenorrhoeic athletes (Thong *et al.*, 2000). This caused NTX to increase by 34% and coincided with reduced oestrogen levels. Ihle and Loucks (2004) were the first authors to show that bone formation is impaired at much higher levels of energy availability than bone resorption is; carboxyterminal propeptide of type 1 procollagen (P1CP) was significantly reduced even at an energy availability of 30 kcal·kgLBM⁻¹·d⁻¹, a level that is not uncommon in endurance athletes (Thong *et al.*, 2000; Fudge *et al.*, 2006). There was however, a different response to energy restriction between the two measures of bone formation; P1CP, which represents type 1 collagen formation, decreased linearly with decreasing energy availability, whereas OC, a measure of matrix mineralisation, decreased the most when energy availability was between 20 and 30 kcal·kgLBM⁻¹·d⁻¹. This suggests that restricted energy availability may affect the bone formation process by various mechanisms, which requires elucidation. However, some field studies have failed to show associations between energy availability and measures of energy conservation (Koehler *et al.*, 2013; Reed *et al.*, 2013). This suggests that other factors that are common in athletic environments, such

as psychological stressors and differences in energy availabilities on different days, (probably due to training schedules), could mean that low energy availabilities do not affect the body's physiological systems in a constant and predictable way, as is often shown during laboratory studies.

VanHeest *et al.* (2014) has shown that ovarian suppression, originating from hypothalamic dysfunction in combination with energy conservation caused by an energy deficit, is associated with poor sports performance, as demonstrated by 400m swim times over a 12 week competitive season. Five elite swimmers that were retrospectively classed as ovarian suppressed, based on serum progesterone and oestradiol levels and self-reported menstrual status, had significant suppression of metabolic and bioenergetic parameters. T3, a thyroid hormone that is suppressed during a sustained energy deficit, was 19% lower in the ovarian suppressed swimmers compared to the 5 swimmers that had cyclic menstrual function. Resting energy expenditure:predicted resting energy expenditure (REE:pREE) was 27% lower and energy availability was 90% lower in the ovarian suppressed swimmers. The authors showed that sports performance declined by 9.8% in the ovarian suppressed group, whereas performance increased by 8.2% in the cyclic menstrual group. The ovarian suppressed athletes expended 900 – 1,225 kcal day⁻¹ during training, with a REE:pREE of 77 – 84%, which is indicative of hypometabolism. This caused energy conservation and therefore maintenance of higher body mass and composition, despite a significant and constant energy deficit. Constant energy deficits in athletes can be maintained for long periods of time, and the results from VanHeest *et al.* (2014) show that this will eventually have detrimental performance effects. It is yet to be investigated whether this also occurs in elite male athletes. This is the first study that has shown a direct performance effect of low energy availabilities in the free-living athlete.

2.7 Methods of measuring energy expenditure

There are numerous methods available to measure energy expenditure in humans; direct and indirect calorimetry and non-calorimetric methods. Non-calorimetric methods include; questionnaires, heart rate monitors, motion sensors and the DLW method. There are a number of strengths and weaknesses of each of these methods which means a compromise is needed, particularly when implementing

these methods in athletes. Ravussin *et al.* (1986) described the use of indirect calorimetry or an open-circuit respiratory chamber and showed that the coefficient of variation (CV) of 24 h energy expenditure was 2.4%, which is similar to other studies (Dallosso *et al.*, 1981; Garby *et al.*, 1984). However, the limitation of participants having to live in a confined chamber for the duration of the study means that this method is inaccessible and unfeasible for the majority of athletes. It also does not represent the normal, daily routines of participants.

The DLW method that was developed by Lifson *et al.* (1955) and validated by Schoeller and van Santen (1982), Schoeller and Webb (1984) and Schoeller *et al.* (1986), allows energy expenditures to be measured in free-living participants over a number of days, without interfering with daily routines. This method involves participants receiving a loading dose of water labelled with ^2H and ^{18}O isotopes, which mix with the hydrogen and oxygen in body water. As energy is expended, carbon dioxide and water are produced; carbon dioxide is only excreted in the breath, but water is excreted in the breath, urine and sweat. As ^{18}O is contained in both carbon dioxide and water, it is lost more rapidly than ^2H is, which is contained in water but not carbon dioxide (Schoeller and van Santen, 1982). Over a period of 4 – 20 days, the difference between the rate of loss of ^{18}O and ^2H from the body reflects the rate at which carbon dioxide is produced. This is then used to estimate energy expenditure using a formula based on carbon dioxide production rate and respiratory quotient (Weir, 1949).

Limitations of this DLW method include the high costs of the isotopes, equipment and expertise needed for analysis and the equations used to determine total energy expenditure and physical activity level. The physical activity level has been calculated from pooled analysis of DLW studies, but it remains unclear whether the equations used to calculate total energy expenditure and estimated energy requirements are appropriate for non-Western populations (Park *et al.*, 2014). Due to the large energy expenditures of athletes and the very different lifestyles compared to the normal population, these equations may not be appropriate for athletes. For example, in a study determining the physical activity level of healthy Japanese adults, the largest value for physical activity level was 1.91 ± 0.30

which represents 1 h of hard muscular work such as training, carrying lumbers or farming (Ishikawa-takata *et al.*, 2008) and athletes will often perform hard training for much longer than 1 h per day.

Despite these limitations, the DLW method is currently considered the gold standard measure of energy expenditure in free-living participants, and as such, there are a number of studies that have employed the DLW method in athletes (Westerberp *et al.*, 1986; Schulz *et al.*, 1992; Sjödin *et al.*, 1994; Trappe *et al.*, 1997; Ebine *et al.*, 2000; Ebine *et al.*, 2002; Ekelund *et al.*, 2002; Hill and Davies, 2002; Fudge *et al.*, 2006; Rehrer *et al.*, 2010). Westerberp *et al.* (1986) measured energy expenditure in 4 cyclists during the Tour de France and showed that the average daily energy expenditure was 8,054 kcal·d⁻¹ when the cyclists covered 3,500 km over 23 days, although it is likely that there was some measurement error due to the length of the study period and the need for multiple DLW doses. Similarly, Rehrer *et al.* (2010) showed that elite cyclists expended 6,548 kcal·d⁻¹ during a 6 day, 10 stage cycling race and the riders managed to match this with energy intakes of 6,525 kcal·d⁻¹ on average, although the authors only collected full data sets for 3 participants and one of the participants did not receive the full DLW dose.

Sjödin *et al.* (1994) showed that elite male cross-country skiers expended 7,218 kcal·d⁻¹ and the females expended 4,374 kcal·d⁻¹ on average during a pre-season training camp, and the energy expenditures were very closely matched with energy intakes. In contrast, Trappe *et al.* (1997) showed that female swimmers expended 5,593 kcal·d⁻¹ but consumed only 3,136 kcal·d⁻¹ on average. Schulz *et al.* (1992) showed that 9 female distance runners expended only 2,768 ± 382 kcal. This is lower than the values reported in a study of elite male Kenyan runners (Fudge *et al.*, 2006), which showed that on average the athletes expended 3,492 kcal·d⁻¹ but consumed 3,165 kcal·d⁻¹. This means that the athletes were in a negative energy balance throughout the study, which took place during an intense training period prior to a major competition. The authors suggest that the accompanying weight loss may be beneficial for performance in these runners as it was gradual and should improve running economy and performance in the heat, especially as the athletes' diets were high in CHO (9.8 g·kgBM⁻¹·d⁻¹), although there were no accompanying measures of metabolic or stress hormones. It is yet to be investigated what effect this gradual reduction in body mass and consistent negative energy

balance in the lead up to a competition has on bone health and bone turnover in runners and other endurance athletes.

2.8 The skeletal system and bone remodelling

The human skeletal system is a robust structure that has many different functions; it protects internal organs, it provides a source of haematopoietic, mesenchymal and endothelial stem cells, it helps regulate essential mineral homeostasis by acting as a reservoir, it gives us shape, structure and it acts as a scaffold to produce movement. Bone is made up of type 1 collagen, consisting of triple helices and crystals of calcium hydroxyapatite [$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$]; type 1 collagen is characterised as osteoid and bone as mineralised osteoid (Gupta *et al.* 2006; Grynopas and Omelon, 2007; Zhu and Prince, 2012). Bone consists of cortical and trabecular bone; cortical bone is the compact bone that is designed to maintain the strength and stiffness of the skeleton and trabecular bone is the cancellous or spongy bone designed to maintain the toughness of the skeleton which has the ability to deform and absorb energy. After skeletal growth or bone modelling is complete, remodelling of bone continues and each year about 10% of the entire skeleton is broken down and replaced with new bone.

Lanyon (1987) showed that in locations where the skeleton has a primary protective role *i.e.*, the skull, the bone's architecture is achieved during growth and is under direct genetic control. However, in parts of the skeleton where resistance to repetitive loading is important, *i.e.*, bones of the lower limbs, only the general shape of the bone will be achieved through growth and genetic control. The remaining characteristics of the bone, such as the stiffness or toughness of the bone, are determined by functional adaptation *i.e.*, mechanical loading placed on the bone, particularly throughout childhood and adolescence. Because the skeleton is in a constant state of repair, these remaining functional characteristics of bone are altered throughout an individual's lifetime. This idea has previously been described as a 'mechanostat', where the mechanism of bone remodelling responds to frequently experienced strains (Frost, 1987). The full sequence of bone remodelling occurs between 100 days and 1 year via a negative feedback loop that involves systemic hormones, such as

IGF-1, GH, calcitonin, vitamin D₃, oestrogen and PTH (Stewart and Hannon, 2000; Zaidi *et al.*, 2002; Nakamura *et al.*, 2007; Krum *et al.*, 2008; Crockett *et al.*, 2011). Although the main function of PTH is to regulate blood calcium levels, it also has an important role in bone formation and prevents osteoblast and osteocyte apoptosis. This is why intermittent administration of PTH is a treatment for osteoporosis, as it acts to increase osteoblast number, bone formation and bone mass (Crockett *et al.*, 2011). PTH will be discussed in more detail in later sections as PTH was investigated in the studies reported in this thesis.

Bone remodelling is defined as an active process throughout the skeleton which requires the sequential and coordinated actions of osteoclasts and osteoblasts that are coupled together at the basic multicellular unit level (Frost, 1964). Osteocytes, which are latent osteoblasts, lie embedded within lacunae in mineralized bone and connect with others via the canicular system, thus forming a cellular network that can respond to mechanical loading and strong forces exerted on the bone (Lanyon, 1998). This means that osteocytes are the main means of bone repair and adaptation to exercise (Frost, 1960; Frost, 1986). For instance, when microdamage or a microcrack is formed during stress fracture development, it causes disruption of the cellular network, this is detected by the osteocytes, which send signals to osteoblasts on the bone surface (Burger *et al.*, 1998). This causes cells from the bone marrow to be incorporated into the area of the microdamage, which then differentiate into osteoclasts and resorb the bone around the microdamage.

Osteoclasts are multi-nucleated, exclusive bone resorptive cells that are members of the monocyte/macrophage family (Suda *et al.*, 1999; Banfi *et al.*, 2010). Osteoclasts resorb bone by firstly adhering to bone, creating a sealing zone into which hydrochloric acid and acidic proteases are secreted. This along with the H⁺ ATPase pump creates an acidic environment that breaks down the bone mineral (Salo *et al.*, 1997; Teitelbaum and Ross, 2003; Stenbeck and Horton, 2004). Additionally, osteocyte apoptosis can occur at sites of microdamage and these dying osteocytes are targeted for removal by osteoclasts (Verborgt *et al.*, 2002).

Osteoblasts are then recruited to the resorption pit where they lay down new osteoid, until the resorbed bone is completely replaced by new. Bone resorption continues for around 2 weeks, the reversal phase, where mononuclear cells appear on the bones surface, may last up to 5 weeks, and bone formation can continue for up to 4 months until the new bone is completely mineralised (Stewart and Hannon, 2000; Hadjidakis and Androulakis, 2006; Crockett *et al.*, 2011) (Figure 5).

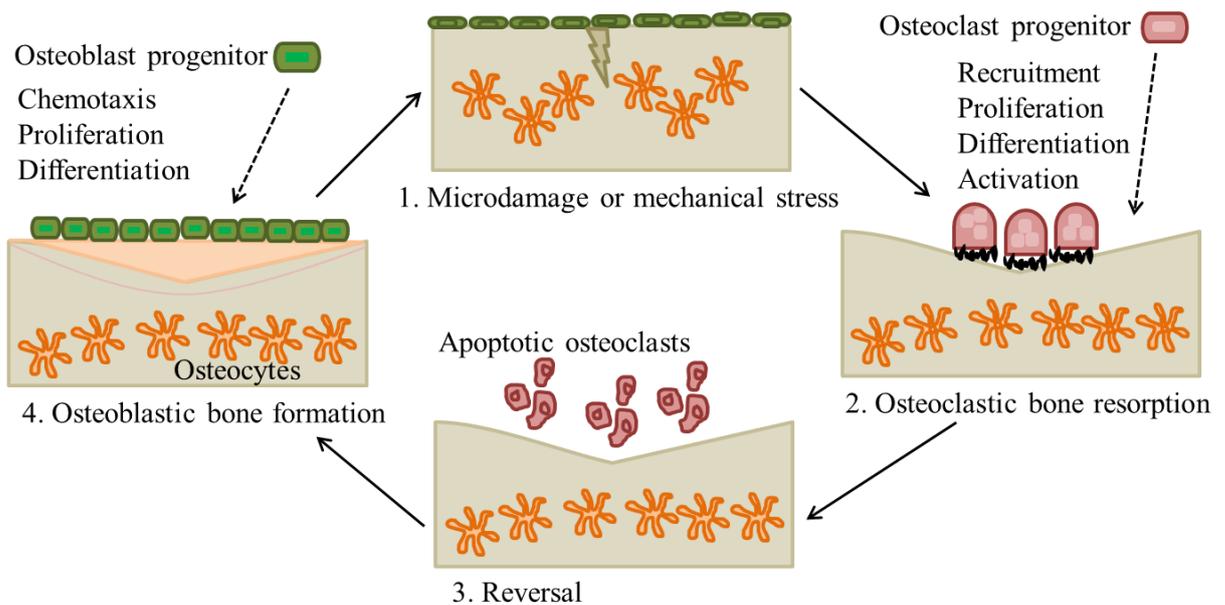


Figure 5. The bone remodelling cycle. Redrawn from Crockett *et al.* (2011). Microdamage or mechanical loading (1) stimulates the recruitment, proliferation, differentiation and activation of osteoclasts that resorb the damaged bone (2). Osteoclasts then die by apoptosis (3), and osteoblasts migrate to the resorption pit by chemotaxis and replace the resorbed bone with unmineralised osteoid, which then becomes mineralised (4).

There are three goals of bone remodelling; 1) to provide a mechanism to maintain homeostasis of essential minerals such as calcium and phosphate, by providing a store that can be released into the serum when needed; 2) to provide a mechanism for the skeleton to adapt to mechanical loading, maintaining skeletal integrity; 3) to provide a mechanism to repair any damage caused by mechanical

loading, that must be repaired to prevent fracture (Parfitt, 1994; Parfitt *et al.*, 2000; Shaffler and Jepson, 2000; Burr, 2002; Frost, 2003). The repair of microdamage is considered the most important purpose of bone remodelling (Heaney, 2003; Parfitt, 2004). At any one time, around 20% of trabecular bone surface is undergoing remodelling (Frost, 1991), but the amount of bone removed and the amount formed should be alike, meaning that the total quantity of bone remains constant under healthy conditions (Currey, 2003). In peripheral trabecular bone, a turnover rate of 2% per year is sufficient to maintain mechanical proficiency of the bone and even after allowing for the other purposes of bone remodelling, bone turnover rates exceeding that may be detrimental to bone health (Parfitt, 2004).

It is hypothesised that there are two types of bone remodelling; stochastic (not site-dependent) and targeted site-specific. The first goal of bone remodelling (mineral homeostasis) can be accomplished via stochastic remodelling, but the second two goals (adaptation to and repair from mechanical loading) require site-specific remodelling (Mori and Burr, 1993; Parfitt *et al.*, 1996; Han *et al.*, 1997; Burr, 2002; Parfitt, 2009). Interventions that prevent the need for stochastic remodelling, without preventing targeted repair of microdamage or adaptation to mechanical loading, would be ideal for athletes training multiple times a day, thus placing significant stress on the skeletal system. This would prevent whole body net bone loss without preventing targeted repair of damaged bone and positive adaptation to altered mechanical loading (Burr, 2002). An example of an intervention that may prevent the need for stochastic bone remodelling is pre-exercise calcium supplementation, which prevents the need to resorb bone to maintain mineral homeostasis (Guillemant *et al.*, 2004; Barry and Khort, 2007; Barry *et al.*, 2011); although, it is not known how such an intervention affects site specific remodelling.

Schaffler (2003) proposed that there is a homeostatic balance between the amount of microdamage occurring and the intrinsic repair by bone remodelling, and when this is not operating properly, *i.e.*, there is too much or too little bone remodelling, microdamage will accumulate and there may be excessive unmineralised bone. When this reaches a threshold, the bone becomes mechanically unstable and will fail, leading to a fracture (Martin, 1992). However, a number of studies that have

examined the bone remodelling threshold have used bisphosphonate treatment in animals to suppress bone remodelling, so it is not currently known what this threshold is in humans or even if this mechanism translates from *in vitro* to *in vivo* (Mashiba *et al.*, 2000; Mashiba *et al.*, 2001; Schaffler, 2003).

As well as diseases such as osteoporosis and arthritis and medications such as bisphosphonates (Allen and Burr, 2011), there are numerous other factors that influence bone remodelling. These can be grouped into mechanical, hormonal or lifestyle factors. Sleep, smoking, energy availability, nutrient ingestion and exercise are examples of lifestyle factors that may affect the amount of bone remodelling in an individual (Ihle and Loucks, 2004; Zanker and Cooke, 2004; Burckhardt *et al.*, 2010; Scott *et al.*, 2012). Calcium and vitamin D intake (Heaney *et al.*, 1982; Lappe *et al.*, 2008; Barry *et al.*, 2011; Tenforde *et al.*, 2015), growth factors (insulin growth factors (IGFs), fibroblast growth factors, transforming growth factors) (Misra *et al.*, 2009), cytokines, macrophage colony-stimulating factor, receptor activator of nuclear factor kappa-B ligand (RANKL), interleukin (IL) - 4, IL-6 (Martin and Sims, 2015), prostaglandins, proteinases (Martin and Ng 1994) and sex hormones (Hetland *et al.*, 1993; Zanker and Swaine, 1998a; Zanker and Swaine, 1998b; Compston, 2001) may also influence bone remodelling, although in reality there are likely to be many more factors that influence bone remodelling, that exceed the scope of this literature review or thesis. Some of these factors, primarily energy availability, nutrient ingestion, exercise, calcium and vitamin D intakes will be discussed in more detail throughout this literature review, as they were key components of this thesis and were measured in the experimental studies.

2. 9 Biochemical markers of bone turnover

Bone turnover refers to the total volume of bone that is both resorbed and formed over a period of time, which can be estimated through the measurement of biochemical markers of bone turnover (Parfitt, 2004). In adults, bone turnover occurs mainly through bone remodelling, but can also occur through bone modelling that mainly occurs during growth (Parfitt, 1996).

Unlike static measures of bone mass and structure, biochemical markers of bone turnover can detect the dynamics of the metabolic status of the bone (Seibel, 2000) and allow for an imbalance in bone turnover to be detected if it exists. But bone turnover markers are only able to estimate whole body bone turnover, rather than a specific rate of bone remodelling (Malm *et al.*, 1993; Maïmoun *et al.*, 2004a; Parfitt, 2004; Jürimäe *et al.*, 2006). An imbalance in bone turnover is often described as an ‘uncoupling’ of bone turnover, where by the concentrations of bone resorption and bone formation markers either change in opposite directions or one changes and the other remains stable. However, bone coupling or uncoupling can only occur at a specific basic multicellular unit. Bone remodelling takes place asynchronously throughout the skeleton at anatomically distinct sites (Sims and Martin, 2014) and this cannot be directly measured *in vivo* as there is no way to isolate the actions of individual basic multicellular units at specific skeletal sites. Therefore measurements of bone turnover markers are not able to measure the level of uncoupling or coupling of bone remodelling, and describing changes in bone turnover marker concentrations as an imbalance of the two processes may be more relevant. The result of uncoupled bone remodelling at the basic multicellular unit level is often a change in bone structure, strength, volume and mass (Seibel, 2000; Seibel, 2005; Ott, 2008; Banfi *et al.*, 2010). Bone metabolism is a phrase that is often used interchangeably with bone turnover and bone remodelling. As previously explained, bone turnover and bone remodelling describe different aspects of bone metabolism. Bone metabolism is used in this thesis to refer to the overall state and the overall bone response to an intervention.

Several blood and urinary markers provide estimations of both bone formation and resorption; the biological processes that govern bone turnover. Caution should be taken when interpreting measured bone turnover markers; the majority of the markers can be found in tissues other than bone, such as cartilage, meaning that concentrations of these markers may be influenced by processes other than bone turnover (Seibel, 2000; Banfi *et al.*, 2010). Structural alterations in bone can occur due to mechanical loading without associated changes in systemic bone turnover markers (Vainionpää *et al.*, 2006; Vainionpää *et al.*, 2009), which may mean that bone turnover markers are not sensitive enough to detect increased or changed bone turnover at the tissue level (Scott *et al.*, 2013). Bone turnover markers are not site specific and do not indicate where on the skeleton the resorption or

formation is taking place, resulting in the assumption that the changes in bone turnover take place at the sites of mechanical loading. Spot measures of bone turnover markers reflect the state of bone turnover at that point in time only or reflect the acute activation of the bone resorption and bone formation processes. Because of the responsiveness of bone turnover markers to nutrient ingestion (Clowes *et al.*, 2002a; Henriksen *et al.*, 2003; Walsh and Henriksen, 2010), exercise (Malm *et al.*, 1993; Kristoffersson *et al.*, 1995; Brahm *et al.*, 1996; Thorsen *et al.*, 1997; Langberg *et al.*, 2000; Guillemant *et al.*, 2004) and other external factors (Vasikaran *et al.*, 2011a), timings of blood samples and the external conditions should be tightly controlled in each instance (Delmas *et al.*, 2000). Day-to-day variability for bone formation markers is between 5 and 13% and for bone resorption markers is between 6 and 35%, under research conditions (Hannon and Eastell, 2000; Blumsohn *et al.*, 1994b).

2.9.1 Bone formation markers

Bone formation markers are direct or indirect products of osteoblast activity that are released into the blood (Delmas *et al.*, 2000), at different phases of osteoblast differentiation and osteoid deposition and maturation. Bone alkaline phosphatase (BALP) is a ubiquitous marker of formation that is found on the outer cell surface of osteoblasts, it is involved in all phases of bone mineralisation and therefore provides a specific indicator of osteoblast activity (Epstein *et al.*, 1988; Van Straalen *et al.*, 1991). There are different isoforms of enzymatic alkaline phosphatase that originate from various tissues such as the liver, bone, intestine, spleen, kidney and placenta. In healthy adults, about 50% of alkaline phosphatase is derived from the liver, with the other 50% arising from bone, whereas during skeletal growth, around 90% of alkaline phosphatase is bone specific (Magnusson *et al.*, 1999). The assays used to measure BALP show up to 20% of cross-reactivity with liver alkaline phosphatase, which can lead to falsely high results (Langlois *et al.*, 1994; Martin *et al.*, 1997; Woitge *et al.*, 1996), and there are therefore more accurate markers of bone formation, that are subsequently discussed.

OC (also known as bone-Gla-protein) is a hydroxyapatite-binding protein, that is synthesised by osteoblasts during bone formation, which can be measured when it enters the extracellular compartment (Poser *et al.*, 1980). OC is also excreted by the kidneys and can be measured in the

urine. The total OC content of the bone accounts for around 15% of the non-collagenous protein fraction and it is considered a specific marker of osteoblast function (Brown *et al.*, 1984). There is also evidence that OC (particularly undercarboxylated OC) plays a role in energy metabolism (Confavreux *et al.*, 2009; Fernandez-Real *et al.*, 2009; Hwang *et al.*, 2009; Confavreux, 2011; Lombardi *et al.*, 2012), therefore, any change in OC during exercise may not be specifically related to changes in bone formation, but could potentially be an adaptation to altering energy requirements that are often changing in athletes (Confavreux *et al.*, 2009). Adding to the limitations of using OC in bone formation assessment, is the presence of various types of OC-derived fragments circulating in the blood stream (intact 1-49 or N-mid 1-43), which are subject to rapid degradation in the serum and this leads to heterogeneity of the OC fragments (Bell, 1997; Seibel *et al.*, 2001; Vasikaran *et al.*, 2011a). Since OC is incorporated into the bone matrix, some OC fragments may also be released during bone resorption, which may be particularly true in individuals with high bone turnover (Chen *et al.*, 1996; Gorai *et al.*, 1997; Salo *et al.*, 1997). Considering the activity levels of the participants studied in this thesis, bone turnover may be higher than average, meaning that OC may not be the best marker of bone turnover in this instance. There are also multiple analytical methods used to measure OC that produce varying results and means that results from one assay cannot be compared to another (Diaz Diego *et al.*, 1994; Masters *et al.*, 1994; Vergnaud *et al.*, 1997; Vasikaran *et al.*, 2011a).

Carboxyterminal propeptide of type 1 procollagen (P1CP) and aminoterminal propeptide of type 1 procollagen (P1NP) are considered to be quantitative measures of newly formed type 1 collagen, as they enter extracellular space following the synthesis of new collagen by osteoblasts (Hassager *et al.*, 1991; Eriksen *et al.*, 1993). Bone collagen consists of 95% of type 1 collagen, and this composes 90% of bone matrix. Type 1 collagen is also found in skin, dentin, cornea, vessels, tendons and fibrocartilage, but most of these tissues exhibit slower turnover rates than bone and therefore they contribute very little to circulating concentrations. Collagen precursors in triple helical form present short-terminal peptides at both the amino and carboxy terminals that are enzymatically cleaved and liberated into the circulation once the precursor molecule is secreted into the extracellular space (Fessler *et al.*, 1975). P1CP has a short serum half-life of only 6 – 8 minutes (Olsen *et al.*, 1977;

Smedsrod *et al.*, 1990) so this is often difficult to capture and measure. P1NP has a longer half-life, there are specific, more accurate (and automated) immunoassays for P1NP and it is thermostable, similar to other markers of collagen metabolism (Seibel, 2000; Seibel, 2005). These are the reasons why this marker of bone formation is measured throughout this thesis.

2.9.2 Bone resorption markers

With the exception of tartrate-resistant acid phosphatase (isoenzyme 5b) (TRAP5b), most bone resorption markers are degradation products of type 1 collagen that can be measured in the blood or urine. TRAP5b is one of five isoforms of ubiquitous acid phosphatases. Isoform 5 has two different subforms; 5a which is expressed by macrophages, platelets and erythrocytes and 5b which is osteoclast specific (Halleen *et al.*, 2000). Osteoclasts secrete TRAP5b into the bloodstream, therefore the concentration and activity of this enzyme represents osteoclast activity (Halleen *et al.*, 1998). TRAP5b is not very stable once blood is drawn; it loses approximately 20% activity every hour (Bais and Edwards, 1976), and this is one of the reasons that TRAP5b is not routinely measured as a marker of bone resorption. Further, the activity of osteoclasts may not directly relate to the amount of bone being resorbed.

RANKL is a member of the tumor necrosis factor ligand family that is involved in the first step essential for activating the bone resorption process by stimulating osteoclastogenesis. When RANKL binds to its receptor; receptor activator of nuclear factor kappa-B (RANK), which is located on osteoclast precursor cells, this causes the differentiation of the osteoclast precursor cells into active osteoclasts. RANKL is expressed on cells of the osteoblastic lineage, particularly immature cells such as osteocytes. Osteoprotegerin (OPG) is a decoy receptor for RANKL, which acts as a paracrine inhibitor of osteoclast formation (Martin and Sims, 2015). When OPG binds to RANKL it prevents the binding of RANKL to its receptor RANK. Thus, the ratio of RANKL/OPG is the more accurate measurement of osteoclast formation and activity, compared to a singular measurement of either receptor. If the RANKL/OPG ratio changes in favour of RANKL, osteoclastogenesis is likely to increase, and if the RANKL/OPG ratio changes in favour of OPG, osteoclastogenesis is likely to decrease (Theoleyre *et al.*, 2004; Kearns *et al.*, 2008). Despite this, true concentrations of RANKL,

OPG and RANK are difficult to measure as they exist in both free and bound forms. RANKL is also undetectable in approximately 70% of healthy controls (Hegedus *et al.*, 2002), which may produce misleading results when expressing the RANKL/OPG ratio (Scott *et al.*, 2010) and is the main reason that RANKL and OPG are not routinely measured as markers of bone resorption.

PYD and DPD function as molecular bridges that cross-link several collagen molecules, providing mechanical stability. Proteolytic enzymes, derived from osteoclast activity, degrade these cross-links and they are subsequently released into the bloodstream and urine. Therefore PYD and DPD reflect degradation of mature, fully mineralised collagen only. PYD is predominately found in cartilage, but is also found in bone, tendon and connective tissue and DPD is found in bone and dentin (Eyre *et al.*, 1988). Due to the much higher turnover of bone compared to the other tissues that PYD and DPD are found in, the PYD and DPD fragments that originate from the other tissues have minimal contribution to the measured concentrations. Despite this, PYD and DPD were previously viewed as the best indicators of bone resorption (Eyre *et al.*, 1988; Seibel *et al.*, 1992; Brixen and Eriksen, 1999; Kraenzlin and Seibel, 1999), but in recent years, assays for cross-linked telopeptide molecules have been developed and are now used more widely.

Cross-linked telopeptides result from the enzymatic degradation of the amino-terminal (NTX) and carboxy-terminal (CTX) regions of type 1 collagen. Following proteolytic degradation, they are released into the blood and then the urine. CTX molecules can be detected as four different isoforms; α -L, β -L, α -D and β -D, that occur due to modifications to the collagen molecules via isomerisation and racemisation, which often occurs as an effect of ageing (Cloos *et al.*, 1998; Gineyts *et al.*, 2000). Bonde *et al.* (1994) developed the β -CTX enzyme-linked immunosorbent assay (ELISA) which was advanced in later years (Bonde *et al.*, 1997). Measurement of β -CTX is now available on automated immunoassay analysers (electro-chemiluminescence immuno assay; ECLIA) which reduce the analytical variability. This is one of the reasons that β -CTX is measured as a marker of bone resorption throughout this thesis, other reasons are explained in subsequent sections.

2.9.3 Variability of bone turnover markers

There are three main causes of variability in the measurement of bone turnover markers; pre-analytical, analytical and biological variability. Standardisation of procedures of all scientific measurements help to control the first two causes of variability, this includes; timing and mode of sample collection, handling, storage and transport of the sample, accounting for thermodegradation and photolysis, preparation for analyses through centrifugation, freezing, thawing and aliquoting (Banfi and Dolci, 2003; Seibel, 2005), as well as using specific and rigorously controlled assays that are part of a routine proficiency testing program (Seibel *et al.*, 2001). Due to large variabilities, small sample sizes are not ideal and serial measurements should be performed whenever possible (Banfi *et al.*, 2010).

Among the different bone resorption markers, breakdown products of type 1 collagen have been shown to have superiority over other degradation products (Garnero *et al.*, 1996b), due to their stability in blood once drawn and due to the development of accurate, automated assays used to measure them (Seibel, 2000; Seibel, 2005; Vasikaran *et al.*, 2011a; Vasikaran *et al.*, 2011b). Serum and plasma based markers have better clinical value than urinary markers because of lower imprecision, diurnal variation, day-to-day variability and intra-individual variability in blood markers compared to urine markers (Popp-Snijders *et al.*, 1996; Garnero *et al.*, 2001). One blood sample is also easier and more convenient than collecting daily urine output, in addition, the correction for creatinine in urine adds another source of variation (Vasikaran *et al.*, 2011a). Christgau *et al.* (2000) and Eastell *et al.* (2000) showed that serum measures of CTX and NTX have an approximately two-fold lower intra-individual variability compared with urinary markers.

Vasikaran *et al.* (2011a) separated sources of pre-analytical variation into “controllable” and “uncontrollable” sources. Controllable sources include; circadian variations, fasting/feeding status, exercise status, menstrual status, seasonal and dietary status. Uncontrollable sources include; age, menopausal status, sex, fracture history, pregnancy and lactation, drugs, disease, immobility, geography, ethnicity and oral contraception use. Some of these factors, such as, sex, fracture history and oral contraceptive use were considered throughout this thesis, whereas some of these factors such as, pregnancy and lactation, drug use and disease were not considered in further sections

because they were not relevant to the participants that participated in the experimental studies of this thesis. These sources of pre-analytical variability were controlled for throughout the experimental studies in this thesis.

The International Osteoporosis Foundation and The International Federation of Clinical Chemistry and Laboratory Medicine recommend that serum or plasma P1NP and β -CTX are used as reference analytes for bone turnover markers in clinical studies (Vasikaran *et al.*, 2011a; Vasikaran *et al.*, 2011b; Vasikaran *et al.*, 2011c), which is another reason that these bone turnover markers are measured throughout this thesis. P1NP and β -CTX are recommended because of adequate characterisation of the markers, the specificity to bone, wide availability of analysis, relatively low biological and analytical variability when sufficiently controlled, ease of sample handling and analysis and sample stability (Vasikaran *et al.*, 2011b).

2.9.4 Circadian rhythms of bone turnover markers

Distinct circadian rhythms of bone turnover markers have been demonstrated in humans. Although markers of bone formation also show circadian rhythms, the relative amplitude of these rhythms is much smaller than that of bone resorption markers (Eastell *et al.*, 1992). Most studies report daily amplitudes of 15 – 30%, but the most pronounced diurnal changes have been shown for CTX (Eastell *et al.*, 1992; Greenspan *et al.*, 1997; Schlemmer and Hassager, 1999; Wichers *et al.*, 1999). Circadian rhythms generally have 2 components; an endogenous component that is controlled by a body clock and is influenced by other endocrine circadian rhythms, and an exogenous component that is influenced by cyclical changes in the external environment, such as diet and exercise. Exogenous influences can amplify endogenous rhythms (Blumsohn *et al.*, 1994b).

DPD has a large-amplitude circadian rhythm, with a peak at around 07:00 and a nadir around 17:00 (Eastell *et al.*, 1992; Schlemmer *et al.*, 1992), and NTX follows a similar rhythm (Blumsohn *et al.*, 1994b). Blumsohn *et al.*, (1994b) showed that daily excretion of DPD and NTX decreased significantly and the circadian rhythm was attenuated in participants that were given a 1000 mg calcium supplement every evening (at 23:00) for 14 days. But excretion did not decrease in

participants that were given the calcium supplement in the morning (at 08:00). This evening calcium supplementation also reversed the night-time increase in PTH.

PTH, which is discussed in more detail later sections, has a pronounced circadian rhythm; there is a peak in PTH in the early hours of the morning between 02:00 and 04:00 and a nadir between 08:00 and 10:00 (Jubiz *et al.*, 1972; Logue *et al.*, 1989; Kitamura *et al.*, 1990; Calvo *et al.*, 1991; Fuleihan *et al.*, 1997). It has been proposed that PTH contributes to the nocturnal increases in bone resorption, as both increase at night (Logue *et al.*, 1989; Calvo *et al.*, 1991; Hassager *et al.*, 1992; Schlemmer *et al.*, 1992). However, Ledger *et al.* (1995) tested this hypothesis in 10 young and 10 elderly participants and showed that when calcium was infused over-night, causing suppression of the peaks in PTH, the nocturnal peaks in NTX persisted. Therefore, the authors concluded that the circadian rhythm of bone resorption is not totally mediated by PTH secretion, but that PTH does set the absolute level of bone resorption at which the circadian rhythm occurs (Ledger *et al.*, 1995). Previous studies have shown that PTH does not respond to feeding of macronutrients (Scott *et al.*, 2012; Sale *et al.*, 2015) but does respond to feeding of micronutrients, such as calcium, PO₄ and vitamin D. The circadian rhythm of PTH is less likely to be controlled by feeding than markers of bone turnover are and is more likely to be endogenous (Fuleihan *et al.* 1997), although Fuleihan *et al.* (1997) failed to consider changes in PO₄ and how they relate to PTH. Logue *et al.* (1992) showed that the daily response of PTH is regulated by the circadian clock throughout a 24 h period and that the timing of the nocturnal peak (02:00 – 06:00) does not change when the sleep pattern is shifted. However, Fraser *et al.* (1994) showed that a 96 h fast significantly alters the circadian rhythm of PTH secretion by lowering serum calcium concentrations and by suppressing the circadian rhythm of serum PO₄, which the authors propose plays an important role in the control of the PTH rhythm.

PYD, OC, BALP, P1CP, CTX and carboxyterminal cross-linked telopeptide of type 1 procollagen (ICTP – a marker of bone resorption) have been reported to have strong circadian rhythms, with peak values all occurring at night/early morning (Gundberg *et al.*, 1985; Hassager *et al.*, 1992; Wichers *et al.*, 1999) and the nadir occurring in the late afternoon (Nielsen *et al.*, 1990; Eastell *et al.*, 1992; Hassager *et al.*, 1992; Schlemmer *et al.*, 1992; Schlemmer *et al.*, 1994; Ledger *et al.*, 1995). The

aetiology of these circadian rhythms is relatively unknown. The nocturnal increase in bone resorption may be caused by the absence of nutrient ingestion and the subsequent decrease in serum calcium levels. Although Schlemmer and Hassager (1999) showed that the nightly fast could only explain a small part of the circadian rhythm in bone resorption and that there is only a significant effect of fasting during the early day time. The circadian rhythm of bone resorption markers is independent of sex, sex hormones, posture, PTH and cortisol (Schlemmer *et al.*, 1994; Ledger *et al.*, 1995; Schlemmer *et al.*, 1997; Schlemmer and Hassager, 1999).

Bjarnson *et al.* (2002) demonstrated that markers of bone resorption do not have a fully endogenous circadian rhythm, but that the variations in concentrations are induced by food intake and are reduced during fasting. Separate intakes of glucose, protein and fat all caused a decrease in bone resorption of a similar magnitude, suggesting insulin is responsible for only part of this response. Bjarnson *et al.* (2002) estimate that a fasting period of more than 4 hours (probably between 6 and 9 hours) is needed for subsequent food intake to induce a decrease in bone resorption. When participants consumed a normal diet, there was a decrease in bone resorption after breakfast (therefore after an overnight fast), but there was no additional decrease in bone resorption after lunch or dinner, which were 4 – 6 hours after the previous meal. The response of serum CTX concentrations during both fasting and normal diet ingestion followed the same pattern, but the increases and decreases were more pronounced and rapid during feeding, so it is unlikely that consuming a meal in the afternoon or evening would cause a suppression of bone resorption markers as it would go against the direction of the natural circadian rhythm.

Clowes *et al.* (2002a) measured bone turnover markers at 09:00 for 10 consecutive days in 20 premenopausal females that were fed or fasted on alternate days. The authors conclude that feeding suppresses all markers of bone turnover (CTX, NTX, DPD, P1NP, OC and BALP); the decrease in bone formation markers was 4% and for bone resorption markers was between 7 and 18%. Likewise, Christgau (2000) demonstrated that fasting reduced the amplitude of the increases and decreases of the circadian rhythm of CTX; the maximum changes from the 24 hour mean were 20.3% in the fasted and 44.8% in the nonfasting. Therefore, it is important to consider nutrient intake in participants

when measuring markers of bone turnover, in order to reduce intra- and inter-individual variability due to circadian rhythms. As such, the timings of all blood samples taken throughout this thesis were controlled for and all resting/baseline samples were taken in a fasted state prior to any physical activity.

2.10 Effects of exercise on bone turnover markers

Studies that have investigated the effects of exercise on bone turnover markers have shown different responses. Additionally, different studies employ very different experimental protocols, which contribute to the lack of detailed understanding surrounding the bone turnover marker response to exercise. Several studies that have investigated the effects of exercise on bone turnover markers have studied sedentary or recreationally active individuals (Brahm *et al.*, 1996; Thorsen *et al.*, 1997; Langberg *et al.*, 2000; Malm *et al.*, 2003; Hermann *et al.*, 2007; Mouzopoulos *et al.*, 2007; Lippi *et al.*, 2008; Scott *et al.*, 2011; Scott *et al.*, 2013), therefore this data may not closely relate to athletes and cannot be simply transferred to elite athletes that train every day.

Research that has studied elite athletes or amateur endurance athletes will be subsequently discussed. A number of investigations have measured bone turnover before and after acute bouts of exercise, that often occur outside the laboratory setting and are often poorly controlled. Data collected in this way, does however have ecological validity and reflects the bone turnover response to a race or training session surrounded by the normal routine of an athlete. This may help provide practical and relevant information about maintaining bone health in athletic populations. Study protocols that take laboratory control of bone turnover measurements into the applied setting of elite athlete training or race environments are therefore necessary.

2.10.1 Bone turnover markers and acute exercise

Crespo *et al.* (1999) studied elite marathon runners that had completed the Marathon World Cup. Blood samples were taken before the race, immediately and 24 h after the race, for the measurement of calcium, PO₄, alkaline phosphatase, TRAP, total proteins and cortisol. The authors showed

significant increases in alkaline phosphatase and decreased TRAP immediately after exercise and 24 hours later. The authors suggest that this means that there was an imbalance in bone turnover in the favour of bone formation, and that the marathon had an osteogenic effect, however, the use of alkaline phosphatase to determine bone formation is dubious as it is a ubiquitous enzyme located in most tissues, and is present in several isoenzyme forms (Harris, 1990). The pre-race sample was taken 5 minutes before the start of the race meaning that this was likely to be after breakfast or other food consumption, which would have affected the variation of the measured metabolites and markers. The authors also failed to control or record food or fluid intake during and after the race, which would have affected the immediate post-race and 24 h post-race measurements.

Malm *et al.* (1993) studied 15 females and 8 males that completed the Helsinki City Marathon. One of the females had undergone a hysterectomy and 2 were on oestrogen replacement therapy, suggesting that they were menopausal, which would have affected bone turnover and bone mass (Garnero *et al.*, 1996a; Vedi and Compston, 1996), therefore these participants should not have been included in the study. OC decreased during and 1 day after the marathon and this decline was sustained for 3 days in males and for the 5 days follow-up in females. BALP activity decreased in females but remained unchanged in males. Hydroxyproline, urinary output of collagen breakdown, increased in both males and females after the race, but this did not reach statistical significance. The authors did not control dietary intakes closely throughout the study period; participants were asked participants to follow a low gelatin diet, which can affect hydroxyproline excretion, and to consume a standardised amount of dairy products each day, although there was no mention of whether participants adhered to this. The authors attribute the changes shown to an increase in cortisol concentrations, which have been shown to increase up to fourfold during a marathon (Fellmann *et al.*, 1989), however cortisol was not measured in this study so this conclusion is speculative.

Langberg *et al.* (2000) measured bone turnover makers in 17 amateur male runners, 7 days prior to, immediately post, 1, 2, 3, 4, 5 and 6 days after completion of a marathon run. Immediately after the marathon run there was a transient decrease in PICP that subsequently increased 1 day after the run, peaked 3 days after and returned to baseline 5 days after the run. ICTP showed a significant increase

immediately after the run, but then returned to baseline 1 day after the run. The immediate post-run changes in P1CP and ICTP suggest an immediate imbalance in bone turnover in the direction of increased bone resorption, but in the recovery period following the race, the direction of the imbalance is reversed and favours increased bone formation. The week of complete rest after the run, is likely to have facilitated the positive bone turnover balance observed. This behaviour is not akin to that of elite runners, who would rarely have a full week of rest. If the participants in this study trained again 1, 2 or 3 days post-marathon, the imbalance in bone turnover in favour of increased resorption may have been maintained. This however needs to be explored as studies that investigate repeated exercise bouts and that reflect normal routines of athletes are lacking. There are a number of limitations of the study, including the lack of a baseline sample taken prior to the marathon run to compare the post-marathon sample. The 'baseline' sample was taken 1 week before the start of the marathon and there could have been significant changes in behaviour in that 1 week period such as, changes in energy and carbohydrate intakes for the purpose of glycogen loading (Coyle, 1991; Hawley *et al.*, 1997).

As well as triathlon and marathon running, ultra-distance running has become more popular in recent years (Hoffman *et al.*, 2010; Knechtle *et al.*, 2011). Mouzopoulos *et al.* (2007) studied 16 male athletes before and after competing in a 245 km ultra-marathon. The authors showed that OC, P1CP and BALP concentrations significantly decreased after the run and ICTP, cortisol and PTH concentrations increased immediately after the run. This suggests that bone formation is suppressed and bone resorption is increased after the completion of an ultra-marathon, which the authors related to an increase in cortisol and PTH concentrations.

Similarly, Kershcan-Schindl *et al.* (2009) studied runners before and after completing a 246 km race. Morning blood samples were taken the day before, immediately after and 3 days after the race, but there were no dietary or exercise controls in place between any of the samples and similar to Langberg *et al.* (2000) the 'baseline' sample was not taken on the same day as the race. The sample taken immediately after the race was taken in the afternoon and it is not clear at what time any of the samples were taken, which is important due to the strong circadian rhythms of the measured markers

(Gundberg *et al.*, 1985; Nielsen *et al.*, 1990; Eastell *et al.*, 1992; Hassager *et al.*, 1992; Schlemmer *et al.*, 1992; Schlemmer *et al.*, 1994; Ledger *et al.*, 1995; Wichers *et al.*, 1999). The authors showed that CTX was significantly increased immediately after the race, as well as 3 days after. RANKL and OPG were increased immediately and 3 days after the race and OC was suppressed immediately after the race but returned almost to baseline 3 days after the race. These results suggest a transient dissociation of bone resorption and formation in the direction of increased bone resorption, but there are several limitations with this study. Although measures of haematocrit were taken, concentrations of the bone turnover markers were not adjusted for changes in haematocrit or plasma volume and considering that the average race time was 32 h 52 min, drastic haemoconcentration was likely to have occurred during this extensive exercise bout (Leithäuser *et al.*, 2003; Wu *et al.*, 2004). Further, the sample taken 3 days after the race allowed time for the participants to rehydrate and correct their fluid balance. This means that the changes in concentrations may not be absolute changes in bone turnover marker concentrations, but they may reflect large decreases and increases in blood plasma volumes and this makes it difficult to draw convincing conclusions from this data.

The lack of agreement in what happens to bone turnover after exercise is not surprising considering the different exercise bouts studied and the lack of control around blood sampling, including timings and dietary control. This is undoubtedly more challenging in a field setting compared to a laboratory setting, as shown in the studies mentioned above, but the basic, necessary controls, that help minimise pre-analytical variation (Vasikaran *et al.*, 2011a), have not been implemented in many of these studies.

Recent investigations published by Scott and colleagues, performed in the laboratory, were more tightly controlled for confounding variables. Scott *et al.* (2010) was the first study to measure bone turnover on the days that follow weight-bearing exercise in a laboratory setting. The authors controlled the dietary intakes of participants throughout the study, prescribing diets for a total of 8 days. Participants also refrained from exercise, other than that performed in the laboratory. Additionally, blood samples were taken after an overnight fast and were always drawn between 08:00 and 08:30. The study investigated the effect of an exhaustive running bout, on markers of bone

turnover, between endurance trained and recreationally active male participants. Times to exhaustion are not stated, but distance covered was greater in the endurance trained participants compared to the recreationally active participants, meaning that the mechanical loading placed on the bones is likely to be different. Despite this, the results showed that there was no significant differences between the endurance trained athletes and recreationally active males for any variable. A rested control group was also included and this group showed no significant changes in any variable throughout the 8 days, which allows the authors to conclude that the changes in bone marker concentrations were due to the exercise undertaken.

The exercise bout performed in the study by Scott *et al.* (2010) caused a significant increase in β -CTX concentrations on follow-up days 1 – 4, OPG increased after 20 minutes and was increased on the first follow-up day. PTH, albumin-adjusted calcium (ACa) and PO_4 were also increased throughout exercise although there was no change in PINP or BALP. The increase in bone resorption that was sustained for at least 4 days, with no subsequent increase in bone formation, suggests that during recovery from exhaustive running, bone turnover favours increased bone resorption. Participants undertook the run in a fasted state which relates closely to eating practices of endurance athletes, as fasted training is often performed (dependent on the phase of the season) for training adaptation purposes (Hansen *et al.*, 2005; Hawley and Burke, 2010; Van Proeyen *et al.*, 2011; Bartlett *et al.*, 2015). The run to exhaustion protocol is likely to have caused further glycogen depletion. Furthermore, participants did not compensate for the energy that they expended during the running bout, and the energy intake remained constant on each day of the experimental protocol. This suggests that participants may have been in a negative energy balance on the exercise day as their energy expenditure is likely to have exceeded their energy intake. This is also relevant to the endurance athlete population, who regularly fail to compensate for large energy expenditures (Fudge *et al.*, 2006; Drenowatz *et al.*, 2012).

Scott *et al.* (2011) compared the effects of exercise intensity on the bone turnover response. The exercise performed was 60 minutes of treadmill running at three different intensities; 55% $\text{VO}_{2\text{max}}$, 65% $\text{VO}_{2\text{max}}$ and 75% $\text{VO}_{2\text{max}}$. Exercise intensity was investigated because it has previously been

shown that military recruits with poor physical conditioning who exercise at higher relative intensities have an increased risk of stress fracture injury compared to relatively fitter recruits (Välimäki *et al.*, 2005). It was therefore hypothesised that cardiovascular intensity, rather than the amount of mechanical loading experienced, may contribute to some of the negative effects of endurance exercise on bone, such as reduced BMD (Bilanin *et al.*, 1989; Hind *et al.*, 2006) and increased stress fracture injury (Bennell *et al.*, 1996b; Lappe *et al.*, 2008). The findings of the study showed that running at 75% VO_{2max} caused a greater increase in β -CTX concentrations compared to running at 55% VO_{2max} in the hour post-exercise, but markers of bone formation were not affected. This suggests that during the hours following a running bout bone resorption is increased but bone formation remained unchanged. However, BALP concentrations increased 3 and 4 days post-exercise, which suggests a more long-term beneficial effect of a 60 minute running bout on bone mineralisation. PTH concentrations were significantly increased after exercise at the highest exercise intensity only, which could not be explained by changes in calcium or PO_4 , this will be explored further in subsequent sections and chapters of this thesis.

2.10.2 Bone turnover and repeated bouts of exercise

The studies discussed in the previous section all measured the effect of acute bouts of exercise on bone turnover, but there is a lack of data surrounding the effect of repeated exercise on bone turnover. This is particularly important for athletes training multiple times a day, as repeated exercise bouts have the potential to cause a greater disturbance in bone turnover than singular acute bouts of exercise (Zanker and Swaine, 2000). Some studies have investigated the effect of prolonged army and navy recruit training programs (8 weeks, 10 weeks and 4 months) on bone turnover and stress fracture incidence or adaptation of the tibia (Lappe *et al.*, 2008; Evans *et al.*, 2009; Izard *et al.*, 2016). Two studies have investigated the effects of 3 or 5 days of consecutive running and energy restriction on bone turnover (Zanker and Swaine, 2000; Ihle and Loucks, 2004), but only one of these studies examined athletes, the other sedentary females.

The lack of research in this specific area may be due to the difficulty in executing this sort of study in a well-controlled manner or the difficulty in accessing athletes that train in this way. There is a

need for more of these types of studies as they reflect the normal routine of endurance athletes more closely and therefore hold more ecological validity. Some endurance athletes such as triathletes, who have 3 disciplines to practice, often train 3 – 4 times a day, leaving minimal recovery time between training sessions and therefore a very limited amount of rest across a whole season. There are currently no studies that have investigated the effects of this type of extreme training schedule on bone turnover.

Scott *et al.* (2013) investigated the bone turnover response to two consecutive bouts of running, with recovery durations of 3 hours or 23 hours between the two running bouts. The different recovery periods did not affect markers of bone resorption or formation during the 1 – 4 days after exercise, nor did the different recovery periods alter the increase in OPG, PTH, ACa and PO₄ in the second bout. One possible reason for the lack of increased bone resorption in the days following exercise could be due to the consumption of a supplementary 660 kcal on exercise days. Participants were provided with a total energy intake of 3,100 kcal, meaning that participants were likely to have maintained energy balance on exercise days and may have been able to fully recover during the 3 hours of recovery between running bouts. This is in agreement with data from Zanker and Swaine (2000) and Ihle and Loucks (2004), who showed that there was no effect of repeated weight-bearing exercise on bone turnover if participants were in an energy balance, but when participants were energy restricted, bone turnover became imbalanced, with suppressed bone formation or increased bone resorption. This suggests that if Scott *et al.* (2013) had not provided participants with the additional 660 kcal to replace the energy expended during the exercise, bone turnover may have become imbalanced to a greater extent in the second exercise bout and during the days following the exercise bout with the short recovery duration.

Zanker and Swaine (2000) reproduced the energy restricted conditions that regularly occur in endurance athletes, but in a laboratory environment. The experimental trials involved 60 minutes of running on 3 consecutive days, where participants consumed either 100% of their estimated energy requirements, or 50% of their estimated energy requirements. P1NP and IGF-1 were reduced by 15% and 17% in response to the energy restricted condition, but there was no change in any bone turnover

markers during the energy balance condition. There was a strong correlation found between the decrease in P1NP and IGF-1 ($r = 0.97$). The authors concluded that in trained male distance runners, who are accustomed to this type of exercise, repeated periods of running do not affect bone turnover, unless the runners are under energy restricted conditions. These findings support that a negative energy balance, reduced levels of IGF-1 and reduced collagen synthesis are linked and that these factors may contribute to the imbalance in bone turnover seen in some trained distance runners (MacDougall *et al.*, 1992; Drinkwater *et al.*, 1993; Hetland *et al.*, 1993).

Oosthuysen *et al.* (2013) investigated the effect of 4 consecutive days of cycling for 3 hours each day in 10 trained male cyclists. β -CTX concentrations increased from pre to post-exercise on days 1 – 2 and BALP concentrations were unchanged, however on days 3 – 4 both β -CTX and BALP concentrations were suppressed pre- to post-exercise. Therefore the third and fourth day of cycling failed to provide an immediate stimulus to promote further bone resorption. Although there was a decrease in exercise intensity and total energy expenditure on day 3, the authors attribute the β -CTX response to a decrease in the rate of calcium lost in sweat, as the β -CTX response reflects the sweat calcium excretion response. The high rate of sweat calcium excretion on day 1 was accompanied by a decrease in serum calcium concentration from pre to post-exercise, but the lower rates of sweat calcium excretion on days 3 – 4 did not affect serum calcium concentrations. The authors failed to explain why the rates of sweat calcium excretion on days 3 – 4 were lower. β -CTX concentrations did not recover to baseline concentrations following 21 h of recovery after each cycling bout, which shows that an extended recovery period, that is longer than one day, is needed for bone resorption to return to baseline levels after prolonged cycling.

This persistent increase in bone resorption may have long-term detrimental consequences on bone health, as the simple presence of more sites of bone resorption is sufficient to increase the weakness of the bone (Parfitt, 1993). This may increase the risk of fracture (Burr *et al.*, 1997), may contribute to low BMD scores in cyclists (Stewart and Hannon, 2000; Rector *et al.*, 2008; Medelli *et al.*, 2009a) and the 60% of cyclists that are classified as osteopenic (Nichols *et al.*, 2003; Rector *et al.*, 2008; Medelli *et al.*, 2009b). Elite cyclists often train for 6 days followed by 1 rest day (Rehrer *et al.*, 2010),

so investigating this routine is pertinent. Hinton *et al.* (2010) measured resting OC, BALP and CTX concentrations throughout a 6 day cycling tour and showed that OC was significantly increased at 1, 3 and 5 days throughout the tour and CTX was significantly increased on day 3. The average resting concentrations of CTX were higher than that of non-active individuals (Appendix A) and there were large individual differences, with one participant having a CTX concentration of approximately 1.8 ngmL⁻¹. Rehrer *et al.* (2010) measured energy intakes in the same cyclists during the same race and showed that there was a decrease in energy intakes from day 2 to day 3 of the tour, which coincided with the increase in CTX in the Hinton *et al.* (2010) paper. This suggests that the total amount of energy consumed on each day may have an acute effect on resting bone turnover on the subsequent morning. Elite triathletes, whose sport involves cycling as well as swimming and running, do not have a rest day each week, and although they may not cycle every day, they will swim, cycle or run every day, therefore investigating the effect of multiple exercise bouts on consecutive days is also pertinent as well as measuring energy intakes during these training days.

Some sports such as football, have a clear beginning and end to a season, with large reductions in training once the season has finished. In football players, markers of bone turnover decreased after 2 weeks of reduced training, but increased again after only 10 days of resumed normal training, indicating that bone turnover markers are sensitive to changes in activity levels throughout a sports season (Karlsson *et al.*, 2003a; Karlsson *et al.*, 2003b). Although triathletes have a competitive season lasting from May until September, they will train all year round with only a few weeks of reduced volumes of training in September/October. This warrants investigations that study athletes throughout a season, to enable any changes in bone turnover due to changes in training loads to be observed.

2.10.3 Bone turnover throughout a competitive season

There are very few studies investigating variations in bone turnover markers in athletes over a season. Maimoun *et al.* (2004b) studied 7 male sub-elite triathletes, at the start of the season and 32 weeks later. At 32 weeks, BMD increased at the lumbar spine and skull, but not at any other sites or for the whole body. The authors attribute this lack of change in BMD to the participants training for an

average of 5 years and therefore their bone mass and architecture had already adapted to the mechanical loading exerted by triathlon training, with the bone adaptation threshold subsequently rising (Casez *et al.*, 1995; Bennell *et al.*, 1997). BALP decreased after 32 weeks but there was no difference in OC or u-CTX, and the authors suggest that there is a suppression of bone formation during a triathlon season. However, drawing this conclusion from a decrease in only one bone turnover marker at one time point is dubious as the singular measurements were too isolated to detect dynamic changes in bone turnover throughout a season. It is likely that perturbations in bone turnover occurred in the 32 weeks between the two samples, as the training load and intensity is likely to have changed, particularly if this coincided with important races. Therefore a singular follow-up measurement is not adequate when monitoring bone turnover, especially in elite athletes.

Jürimäe *et al.* (2006) examined the effect of a 6-month heavy training period in elite and sub-elite rowers, on BMD, BMC, markers of bone turnover and related hormones. However, these authors also made only 2 measurements; one after a relative rest period where the training volume was 11.6 ± 0.4 h per week and one after a 6-month heavy volume period where the training volume was 16.8 ± 0.6 h per week. The authors showed an increase in OC, which was the only bone turnover marker measured. OC may not be specifically related to changes in bone formation, but could potentially be an adaptation to the altering energy requirements of the rowers (Confavreux *et al.*, 2009), which is likely to have increased due to a 5 h increase in training each week. However, there were no measures of energy intakes or energy expenditures reported to help elucidate whether this was the case. IGF-1 and the IGF-1 index increased significantly after the 6 months of high volume training and they were significantly correlated. The apparent relationship between OC and IGF-1 is supported by Canalis (1996) and Zanker and Cooke (2004), who advocate the link between energy balance, IGF-1 and bone formation in athletes and females with anorexia nervosa. Whole body BMD remained unchanged in the rowers, with only arm BMD increasing. This increase in arm BMD, without alteration of whole body BMD supports the notion that increased bone formation occurred at the specific skeletal site that was subject to mechanical loading, but that this was not great enough to transfer to whole body increases in bone formation. Again the main limitation of this study is the two isolated measures of only one bone turnover marker.

Lombardi *et al.* (2011) measured BALP, OC, β -CTX and TRAP5b in 14 elite female skiers, at 3 different phases of training; after a relative rest period (T1), after the pre-competition season (T2) and after the competition season (T3). BALP, OC and TRAP5b increased from T2 to T3. Increased concentrations of both bone resorption and formation markers demonstrated higher bone turnover during an intensive competition period. This increased bone turnover may be due to increased mechanical loading, caused by a demanding competition schedule. Lombardi *et al.* (2011) describe the limitations of measuring bone turnover markers in athletes and comparing the values to established 'normal' ranges. The constant physiological stress and high level of mechanical loading experienced by athletes induces greater perturbations in bone turnover and other physiological responses, compared to the non-active population. However, normal ranges of bone turnover markers have not yet been established in athletes for specific sports. This would be advantageous, as every sport exerts a unique amount of mechanical loading and stress on the athletes' skeletal system. If a universal assessment schedule was implemented, a similar assessment schedule to this investigation would be ideal to establish normal ranges of bone turnover markers in specific athletes. This would allow investigators to assess changes over time in relation to different training phases and would allow for the comparison between athletes of different sports.

2.11 Calcium

It is well known that calcium is important for bone health and the skeletal system. In bone, calcium exists as hydroxyapatite, which influences bone strength and mass. Calcium intake is considered a major modifiable environmental factor in maximising peak bone mass during growth and reducing bone loss during later life (Specker, 1996; Zhu and Prince, 2012). Specker (1996) concluded that there is only a beneficial effect of physical activity on bone health if calcium intakes exceed 1000 mg·d⁻¹, and that the benefits of a high calcium diet only exist when an individual performs physical activity, *i.e.* the two factors do not work independently of each other. The benefits of calcium and vitamin D intake are well documented in elderly populations, with regards to preventing bone loss

and fractures and there is increasing evidence of this in athletes (Medelli *et al.*, 2009a; Nieves *et al.*, 2010; Tenforde *et al.*, 2010; Sonnevile *et al.*, 2012).

The recommended daily allowance of calcium is 700 mg for healthy adults, 800 mg for adolescent females and 1000 mg for adolescent males, females in the menopause and those with osteoporosis (British Dietetics Association and the National Health Service). There are no specific recommended intakes for athletes, but due to the large amounts of physical activity performed by athletes, the requirement for calcium in athletes is likely to be higher than that for non-active healthy adults (Kunstel, 2005).

Calcium intake may modulate the circadian rhythm of bone resorption markers. Blumsholn *et al.* (1994a) showed that when premenopausal women ingested 1000 mg of calcium at 23:00, the night time increase in PTH was abolished and the circadian rhythm of bone resorption (DPD and NTX) was attenuated, but this effect did not transcend into the next day. When the same amount of calcium was given at 08:00, there was no change in the circadian rhythm of the bone resorption markers and PTH was only suppressed in the day but not at night time. The results of this study suggest that the daily rhythm of bone resorption may be determined in part by calcium intake and PTH secretion.

It is well established that an acute ingestion of calcium rapidly suppresses bone resorption (Reid *et al.*, 1986; Reginster *et al.*, 1993; Blumsholn *et al.*, 1994a; Horowitz *et al.*, 1994; Yang *et al.*, 1994; Rubinacci *et al.*, 1996), however these studies used calcium salts rather than dairy products. Dairy products contain additional micro- and macronutrients that may slow down calcium absorption and digestion rates. However, Haakonssen *et al.* (2014) showed that a dairy based, pre-exercise meal did not affect gut comfort or performance in cyclists, thus consuming real dairy products may be preferred to calcium salts in athletes as they provide other essential micro- and macronutrients.

Green *et al.* (2003) tested skimmed milk vs calcium phosphate and showed that the skimmed milk caused a decrease in CTX, despite no significant changes in serum calcium or PTH. Calcium phosphate caused significant changes in serum calcium and PTH, as well as a decrease in CTX in

the first few hours after ingestion. The authors attribute this to the calcium phosphate having a faster transit time through the gut, whereas milk clots in the stomach and gastrointestinal tract meaning its digestion is slower. The authors failed to discuss the addition of PO_4 to the calcium salt which would affect PTH, or the reason why the skimmed milk caused a decrease in CTX concentrations but did not affect serum calcium or PTH concentrations. This could be due to the lactose, protein or fat contained in the milk causing the release of post-prandial and gastrointestinal hormones that may contribute to CTX suppression (Clowes *et al.*, 2003).

Guillemant *et al.* (2004) studied 12 trained triathletes and demonstrated that when 60 minutes of cycling was performed at 80% $\text{VO}_{2\text{max}}$, without a calcium load before or during the exercise, CTX concentrations increased progressively 30 minutes after the start of the exercise and two hours after the exercise, concentrations were still significantly elevated by 45 – 50%. Similarly, PTH concentrations increased 2.5 – 3 fold during the exercise but returned to baseline concentrations 30 minutes after the completion of the exercise. The authors attribute this PTH response to the loss of calcium in sweat; dermal calcium losses may cause a decrease in serum calcium, which means PTH is secreted to rectify this decrease. In contrast, when participants consumed a total of 972 mg of calcium, in fractionated amounts every 15 minutes, from 2 hours before exercise until the end of exercise, the increase in CTX concentrations was completely suppressed and the increase in PTH was partially suppressed. BALP concentrations fluctuated and showed no significant changes with or without calcium intake. The authors concluded that fractionating the intake of high calcium water may be a method to prevent deviations from calcium homeostasis and that the burst of osteoclastic activity that is induced by an acute bout of cycling can be suppressed by the previous intake of a calcium load.

Barry *et al.* (2011) hypothesised that disruption of calcium homeostasis is a potential mediator of bone loss, which is illustrated in Figure 6. This hypothesis is based on the findings that sweating during exercise can cause large dermal calcium losses (Klesges *et al.*, 1996; Barry and Kohrt 2007; Barry and Khort 2008); Chinevere *et al.* (2008) suggested that mineral losses in sweat could contribute up to 40% of the daily recommended intake of calcium. Dermal calcium loss may cause

a decrease in Ca^{2+} concentrations, leading to increased PTH and increased in bone resorption. If dermal calcium losses are replaced by oral calcium supplementation this may attenuate the increase in bone resorption.

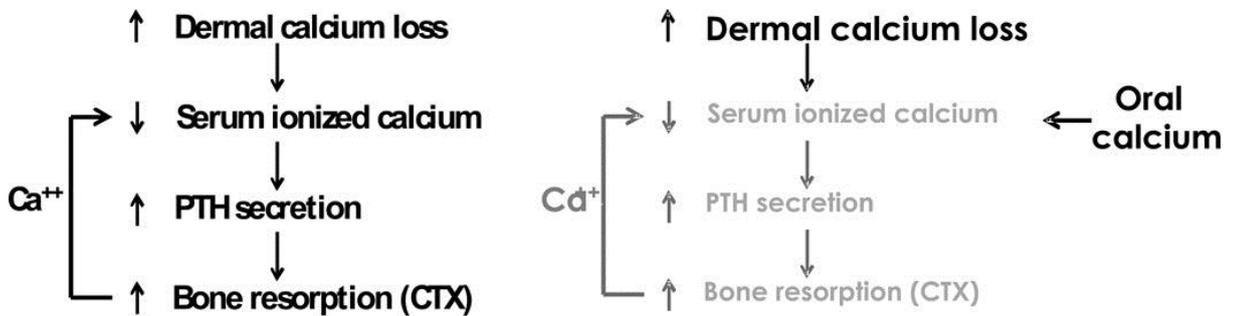


Figure 6. Reproduced with permission from Lippincott Williams and Wilkins and the American College of Sports Medicine; Barry *et al.* (2011). This figure shows how dermal calcium loss may cause increased bone resorption, mediated by decreased Ca^{2+} and increased PTH concentrations. If dermal calcium losses are replaced by oral calcium supplementation this may attenuate the increase in bone resorption. Ca^{2+} (ionised calcium) PTH (parathyroid hormone), CTX (carboxyterminal cross-linking telopeptide of type 1 collagen).

Barry *et al.* (2011) tested this hypothesis by supplementing 20 male endurance athletes during 35 km cycling time trials, with differing calcium supplementation conditions. The three trials consisted of; 1) ingesting 1000 mg of calcium before exercise and ingesting placebo during exercise; 2) ingesting placebo before exercise and 1000 mg throughout exercise; 3) ingesting placebo both before and during exercise. The authors showed that compared to the placebo condition, calcium ingestion before exercise significantly attenuated the increase in PTH during exercise and there was a similar trend for calcium ingestion during exercise; however there was no change in CTX, BALP or Ca^{2+} . Measurements of Ca^{2+} every 15 minutes may not have been regular enough to observe changes, as PTH is secreted within seconds of a change in Ca^{2+} and concentrations are therefore quickly normalised (Brown, 2000). The authors assume that calcium supplementation attenuates the increase in PTH due to the increased availability of non-skeletal calcium to be absorbed from the gut,

minimising the extent to which skeletal stores need to be mobilised. Haakonssen *et al.* (2014) also showed that consuming a calcium rich breakfast (1,350 mg) 90 minutes before a prolonged and high intensity bout of cycling attenuates the increase in PTH and CTX and maintains Ca^{2+} in elite female cyclists.

The amount of sweat and dermal calcium loss is probably dissimilar across sports due to differing environmental conditions, heat acclimation and individual differences (Chinevere *et al.*, 2008), which has never been investigated in elite athletes. Chronic and profuse sweating may increase the chance of mineral deficiencies when intakes are not sufficient to replace the mineral losses in sweat (Chinevere *et al.*, 2008) and in accordance with this, some studies have shown lower levels of plasma minerals in athletes compared to controls (Haralambie, 1981; Campbell and Anderson, 1987; Nishiyama *et al.*, 1996).

2.12 Parathyroid hormone

PTH is a calcitropic hormone that is secreted by the parathyroid gland to defend against a low serum calcium concentration. PTH secretory activity is regulated by the presence of Ca^{2+} in the serum, which is detected by the calcium-sensing receptor on the parathyroid gland (Brown, 1983; Brent *et al.*, 1988; Brown, 2000). $1,25(\text{OH})_2\text{D}_3$ is the second major Ca^{2+} elevating hormone (Brown, 2013). Vital functions of Ca^{2+} involve maintaining plasma membrane integrity, it is present in adhesion molecules, clotting factors and enzymes (Brown and MacLeod, 2001), it activates exocytosis, action potentials and the contraction of muscles (Hofer and Brown, 2003), it is also critical, along with PO_4 , to the mineral component of the skeleton (Bringhurst *et al.*, 1998; Pietrobon *et al.*, 1990). Therefore, maintaining adequate serum concentrations of Ca^{2+} is vital.

When serum calcium levels drop in resting humans, PTH is secreted within seconds and acts to increase serum calcium levels by mobilising calcium from bone reservoirs, by stimulating osteoclasts via a cascade of signals within the bone to induce bone resorption and by increasing renal and intestinal absorption of calcium, mediated by $1,25(\text{OH})_2\text{D}_3$ (McSheehy and Chambers, 1986;

Thorsen *et al.*, 1997; Brown 2000; Zitterman *et al.*, 2002; DeLuca, 2004; Brown, 2013). When Ca^{2+} decreases rapidly, as it may do during intense exercise, there is a more vigorous PTH response than when Ca^{2+} falls slowly (Diaz *et al.*, 1999). PTH and calcitonin, which works in the opposite way to PTH, are regulatory hormones that are involved in a self-limiting, negative feedback loop, that sets the homeostatic level of Ca^{2+} (Brown, 2000).

PTH exerts its effects on bone resorption indirectly via the osteoblast (Qin *et al.*, 2004). Studies have shown that various modes of PTH administration regulate the RANKL:OPG ratio differently, which determines the degree of osteoclast differentiation and activation (Lacey *et al.*, 1998; Yasuda *et al.*, 1998), therefore affecting the amount of bone resorption. Ma *et al.* (2001) showed that continuous infusion of PTH in rats caused a decrease in messenger ribonucleic acid (mRNA) for OPG and an increase in mRNA for RANKL, suggesting that this favours activation of osteoclasts. In contrast, when PTH was administered intermittently, changes in osteoclast activity and bone resorption were not observed. Therefore, PTH has the potential to have both anabolic and catabolic effects on the skeletal system and the twofold effects of PTH seem to be primarily reliant on the duration of elevation above baseline levels, and secondly the concentration of PTH (Tam *et al.*, 1982; Frolik *et al.*, 2003).

Frolik *et al.* (2003) suggested that in order to create an anabolic bone response, the duration of PTH exposure should be kept to a minimum, but the absolute concentration of PTH may be less important. Furthermore, when PTH is administered, prolonged elevation as a result of continuous infusion has been shown to cause bone loss (Tam *et al.*, 1982; Hock and Gera, 1992; Uzawa *et al.*, 1995) and transient spikes as a result of repeated injections or a daily injection result in a net gain in bone mass (Reeve *et al.*, 1981; Tam *et al.*, 1982; Dempster *et al.*, 1993; Riond *et al.*, 1998; Dempster *et al.*, 2001). Tam *et al.* (1982) attribute the anabolic effect to the 1-34 region of the PTH molecule and suggest that PTH stimulates the formation of bone independently of the resorptive effects of PTH. Dempster *et al.* (2001) showed that the anabolic actions of PTH injections affect both cortical and trabecular bone of the iliac crest and that daily injections caused an increase in BMD and parameters related to bone strength, in osteoporotic men and women.

Chronic elevation of PTH concentrations, caused by prolonged strenuous exercise such as endurance training, can lead to a state of moderate secondary hyperparathyroidism (Ljunghall *et al.*, 1986; Ljunghall *et al.*, 1988). This can cause an increase in bone resorption, bone loss and a loss of the circadian rhythm of PTH (Tsai *et al.*, 1984; Chappard *et al.*, 2001). On the other hand, Vainionpää *et al.* (2009) has shown that exercise training can decrease basal PTH levels, which would have the effect of increasing the difference between PTH levels when at rest and when they are increased during exercise. This greater relative increase in PTH has been shown to have the maximum anabolic effect on bone (Brahm *et al.*, 1997a; Brahm *et al.*, 1997b) and this anabolic environment may be induced with short bouts of high-intensity exercise.

Estepa *et al.* (1999) showed that an acute elevation in PO_4 stimulated PTH secretion even when Ca^{2+} concentrations were maintained at baseline, but the increase in PTH was not rapid and took 30 minutes to respond. The PO_4 concentrations needed to stimulate PTH exceeded the range of normal diurnal variation, but it is unknown whether the increased PO_4 concentrations reached during exercise will also stimulate PTH secretion, independent of Ca^{2+} . In contrast, Martin *et al.* (2005) showed that stimulation of PTH release from the parathyroid glands by PO_4 occurred rapidly within 10 minutes and was independent of changes in calcium. As transient spikes in PTH concentrations can have anabolic effects on bone, manipulating the PTH circadian rhythm could be a possible method to improve bone health; with PO_4 used to stimulate an increase in PTH at night-time and calcium used to decrease PTH secretion in the morning, thus exaggerating the transient peaks in the endogenous PTH rhythm (Fraser *et al.*, 2004; Martin *et al.*, 2005). If this was implemented around exercise it could amplify anabolic conditions for bone, but it is not known whether the exercise induced increases in PTH will have the same anabolic effect as exogenous PTH injections. The injections cause an increase in PTH when serum calcium is likely to be stable with no need for increased bone resorption. Alternatively, if an increase in PTH during exercise is triggered due to a decrease in serum calcium, calcium is therefore unstable and there is a metabolic need for increased bone resorption. Further studies are needed to elucidate the effect of exercise-mediated increases in PTH on bone health (Barry and Khort, 2007).

2.12.1 Parathyroid hormone in response to exercise

Studies examining the effects of exercise on serum calcium and PTH are inconsistent; some studies show an increase in serum calcium with exercise (Zerath *et al.*, 1997; Crespo *et al.*, 1999, Takada *et al.*, 1998; Scott *et al.*, 2010; Scott *et al.*, 2011; Scott *et al.*, 2013) and others have shown a decrease (Barry and Khort, 2007; Boussida *et al.*, 2003; Maïmoun *et al.*, 2005; Thorsen *et al.*, 1997) or no change (Maimoun *et al.*, 2006; Scott *et al.*, 2012; Scott *et al.*, 2014). It is therefore not clear whether the increase in PTH in response to exercise is controlled by a decrease in serum calcium concentrations and this requires clarification (Barry and Khort, 2007).

High or moderate exercise intensities induce significant increases in PTH and lower exercise intensities do not induce any significant changes (Maimoun *et al.*, 2006; Barry and Khort, 2007; Herrmann *et al.*, 2007; Scott *et al.*, 2011). Maimoun *et al.* (2006) showed that 50 minutes of cycling at 15% above the ventilatory threshold transiently increased PTH secretion, along with total calcium, PO₄, BALP, OC and CTX, but 50 minutes of cycling at 15% below the ventilatory threshold did not cause an increase in PTH. Similarly, Scott *et al.* (2011) showed that 60 minutes of running at 55% and 65% VO_{2max} did not induce any significant changes in PTH during exercise. Conversely, running at 75% VO_{2max} induced a significant increase in PTH concentrations during exercise, with a maximum increase of 84% from baseline concentrations at 60 minutes. The reason for these different responses and what controls them is unknown, but it may be due to different changes in serum calcium and PO₄ concentrations, as these control changes in PTH at rest (Brown, 2000; Tfelt-Hansen, 2005). It should be noted that these studies used different types of exercise (cycling and running), which should be acknowledged when interpreting the results of different studies due to differences in mechanical loading between activities.

Scott *et al.* (2010, 2011 and 2013) monitored PTH levels for 3 hours following endurance exercise, and decreases in PTH concentrations below baseline were observed, which was also shown by Guillemant *et al.* (2004). These post-exercise changes in PTH may be related to its circadian rhythm; there is a nadir between 08:00 and 10:00 (Jubiz *et al.*, 1972; Logue *et al.*, 1989; Kitamura *et al.*,

1990; Calvo *et al.*, 1991; Fuleihan *et al.*, 1997) and the rapid decreases shown after exercise coincide with this nadir in the late morning (Fuleihan *et al.*, 1997; Scott *et al.*, 2010; Scott *et al.*, 2011; Scott *et al.*, 2014). However, Scott *et al.* (2014) recently showed that post-exercise PTH concentrations were lower than those in a non-exercising control group, suggesting a genuine role of exercise in reducing PTH concentrations below pre-exercise levels.

Scott *et al.* (2013) showed that when two exercise bouts are performed 3 hours apart, the PTH response in the second bout is not altered by the first bout; PTH concentrations increased from baseline by approximately 69% in both bouts. Conversely, Bouassida *et al.* (2003) showed that when a second exercise bout was performed 40 minutes after a first exercise bout (protocol 2), there was no increase in PTH in the second bout. This is surprising as the relative exercise intensities of the first and second exercise bouts were 70% and 85% of VO_{2max} and it has been shown that higher exercise intensities cause greater increases in PTH concentrations (Maïmoun *et al.*, 2006; Scott *et al.*, 2011). When the exercise bouts were combined and were performed with no recovery in between (protocol 1), the second half of the exercise caused an 85% increase in PTH concentrations compared to only a 15% increase in the first half. These results suggest that there may be a minimal time for recovery in between exercise bouts (between 40 minutes and 3 hours) that allows the parathyroid gland to 'reset' and respond to the second exercise bout (Scott *et al.*, 2013).

The discrepancy between the results of the two studies could be due to the duration of the exercise bouts. In the investigation by Scott *et al.* (2011) the exercise bouts consisted of 60 minutes of running at 65% VO_{2max} , whereas in the investigation by Bouassida *et al.* (2003) the exercise bouts were 21 minutes of running at 70% and 85% VO_{2max} . The lack of studies using similar exercise bouts makes it difficult to accurately compare the PTH response to exercise. However, the conclusions of the study by Bouassida *et al.* (2003) should be interpreted with caution. Participants ate breakfast before each exercise trial but there is no mention of whether this was controlled or repeated in each trial. The sampling protocol used is also questionable; there was a blood sample at the end of the exercise bout in protocol 1 (42 min) but there wasn't an equivalent sample at the same time in protocol 2, nor was there a sample at 82 minutes in protocol 1. As the PTH and Ca^{2+} responses followed a similar

pattern in both protocols in the samples that were taken, it is feasible that if samples were taken at those missing time points, the PTH and Ca²⁺ responses could actually follow a very similar pattern in both protocols. Further, the authors compared samples at the end of exercise in both protocols, but these were at 42 minutes or 82 minutes into the trials and were therefore at different times of the day.

The PTH response to exercise may also be different depending on the training status of individuals, however Scott *et al.* (2010) showed no differences between endurance trained and recreationally active males for any variable, including PTH, Aca, PO₄, OPG, BALP, PINP or β-CTX. Likewise, Nishiyama *et al.* (1988) showed no effect of training status on the PTH response to exercise, but the authors speculated that athletes have a higher overall bone turnover compared to non-active individuals.

2.13 Nutrient ingestion and bone

Human bone collagen synthesis has been described as a rapid, nutritionally modulated process that occurs at the same rate as muscle protein turnover. Intravenous feeding with glucose, lipids and amino acids can increase bone collagen synthesis by approximately 66% (Babraj *et al.*, 2005). Diet plays a dual role in regulating bone turnover; not only does nutrient ingestion provide the substrates for bone collagen synthesis, it also causes the release of crucial hormones that play a part in the regulation of bone turnover. The response of bone turnover to feeding and the endocrine mediators of this response will be discussed in this section.

2.13.1 Mixed nutrient ingestion

It is well documented that fasting reduces the circadian variation of β-CTX (Schlemmer and Hassager, 1999; Christgau *et al.*, 2000). Schlemmer and Hassager (1999) investigated a 33 hour fast on the circadian variation in markers of bone turnover in 11 premenopausal women, compared to a control period where regular meals were consumed. The authors showed that there was a significantly less pronounced variation in u-CTX and PTH in the fasting condition compared to the control condition, however there was no change observed in OC by fasting. Similarly, Christgau *et*

al. (2000) showed that fasting in 15 postmenopausal women significantly reduced average circadian variation of CTX from 36% to 8.7%. These results suggest that circadian rhythms of bone resorption markers are not completely endogenous but are partly controlled by food intake.

Feeding with a mixed meal, calcium, glucose, protein or fat transiently suppresses bone resorption at rest (Blumsohn *et al.*, 1994a; Bjarnason *et al.*, 2002; Clowes *et al.*, 2002a; Clowes *et al.*, 2003; Henriksen *et al.*, 2003). Clowes *et al.* (2002a) observed a 19% decrease in β -CTX following a mixed meal, Bjarnason *et al.* (2000) showed a 55% decrease in β -CTX concentrations after a 75g oral glucose load and Henriksen *et al.* (2003) showed a 52% decrease in β -CTX concentrations after a 75g oral glucose load. Bjarnason *et al.* (2002) showed that separate intakes of glucose, protein and fat all led to a similar decrease in β -CTX concentrations, suggesting that a factor other than insulin that is secreted in the postprandial phase may cause or contribute to the decrease in β -CTX. This could be a number of gastrointestinal hormones, as the suppression of β -CTX is greater after oral intakes of nutrients compared to intravenous feeding (Bjarnason *et al.*, 2002; Clowes *et al.*, 2003).

2.13.2 Endocrine mediators

It is likely that there are several mediators of the acute bone turnover response to feeding, but the mechanism of the nutrient-induced suppression of bone resorption is unknown (Clowes *et al.*, 2003; Walsh and Henriksen, 2010). Calcitropic hormones (PTH and calcitonin), GH, IGF-1, cortisol and insulin are not likely mediators of the acute response to feeding, but intestinal and pancreatic peptides such as glucagon-like peptide-2 (GLP-2) could be potential mediators of the post-prandial regulation of bone turnover (Clowes *et al.*, 2003; Henriksen *et al.*, 2003; Walsh and Henriksen, 2010).

Clowes *et al.* (2003) used octreotide, an analog of somatostatin that inhibits the postprandial secretion of intestinal and pancreatic peptides, including insulin, glucagon, gastrin, calcitonin, glucose-dependent insulinotropic peptide (GIP), glucagon-like peptide-1 (GLP-1), GLP-2 and pancreatic polypeptides (Lamberts, 1988), to clarify the mechanism of nutrient induced suppression of bone resorption. The authors showed that octreotide completely abolished the suppression of bone resorption from an oral glucose intake. The mechanism mediating this effect remains unclear, but

the authors suggest that there may be an entero-osseous interaction between gastrointestinal hormones and bone turnover. Enteroendocrine hormone receptors have been identified on osteoblasts and osteoclasts and have been shown to modulate their activity *in vitro* and *in vivo* at pharmacological doses (Cornish *et al.*, 1998; Haderslev *et al.*, 2002). Likewise, Li and Mühlbauer (1999) showed that food fractionation increased BMD and reduced bone resorption in rats, suggesting that increased secretion or increased frequency of secretion of enteroendocrine hormones may have a positive effect on bone turnover.

Henriksen *et al.* (2003) studied the release of GIP and GLP-2 after ingestion of glucose, fat, protein and fructose. The authors further investigated parenteral administration of GIP and GLP-1 and GLP-2 at different doses. The results of the study showed that only GLP-2 was secreted with glucose and protein ingestion and was in parallel with the suppression of β -CTX. This excluded GIP and GLP-1 as key mediators of the acute bone turnover response to feeding. Additionally, subcutaneous injections of GLP-2 caused a significant and dose-dependent reduction in β -CTX. The authors advocate that GLP-2 could be the link in the entero-osseous axis. The link may be related to afferent nerve fibres in the myenteric ganglia (Bjerknes and Cheng, 2001), could have a direct effect on osteoblasts and osteoclasts or it could be related to cytokines that inhibit osteoclast function (Henriksen *et al.*, 2003). Furthermore, Henriksen *et al.* (2004, 2007 and 2009) showed that subcutaneous injections of GLP-2 caused dose-related reductions in β -CTX and increased or unchanged OC and P1NP levels, suggesting that GLP-2 can alter bone turnover in favour of bone formation. However, studies that have measured GLP-2 in relation to feeding and exercise have shown that GLP-2, as well as leptin and ghrelin are unlikely mediators of the effect of feeding on bone turnover when this is combined with exercise (Scott *et al.*, 2012; Sale *et al.*, 2015).

Fat and protein have been shown to have little effect on insulin secretion (Collier and O'Dea, 1983; Nuttall *et al.*, 1984; Bjarnson *et al.*, 2002), but still cause a decrease in β -CTX, which excludes insulin as a key mediator of the acute regulation of bone turnover. Clowes *et al.* (2002b) showed that hyperinsulinemia did not result in a significant change in bone turnover. Despite this, insulin could have longer term effects on bone formation and mass (Walsh and Henriksen, 2010). Cortisol

secretion does not change in response to an oral glucose load at rest (Tse *et al.*, 1983) or with exercise (Sale *et al.*, 2015), so is unlikely to mediate the bone turnover response to feeding. Due to time courses of changes in GH and IGF-1 after feeding, it is unlikely that they are mediators of the acute postprandial response of bone turnover, but are likely to be medium-term regulators of bone turnover, especially when individuals are in an energy balance (Tse *et al.*, 1983; Thissen *et al.*, 1994; Walsh and Henriksen, 2010). Similarly, ghrelin is likely to be a medium to long-term mediator of bone turnover, via its actions on sympathetic tone (Yasuda *et al.*, 2003).

2.14 Exercise related feeding practices

Scott *et al.* (2012) compared the effect of an overnight fast with feeding of a mixed meal on the bone turnover response to 60 minutes of treadmill running at 65% $\text{VO}_{2\text{max}}$. Feeding in the morning before the exercise bout reduced β -CTX concentrations prior to exercise below those in the fasted condition, but by 1 hour post exercise β -CTX concentrations had risen and were similar in both conditions. By 3 hours post exercise β -CTX concentrations had significantly reduced in the fasted condition below baseline and concentrations in the fed condition. Concentrations of β -CTX were elevated above baseline in the fed condition for 4 days post exercise. There was no change in bone formation markers throughout both trials. Scott *et al.* (2012) suggest that the failure of pre-exercise feeding to prevent the subsequent increase in β -CTX may have been the result of the stimulatory response of PTH on β -CTX overriding the effect of feeding, as PTH concentrations increased by 70 – 90%. The increase in β -CTX post-exercise may be the early phase of a PTH-mediated anabolic effect on bone (Zikan and Stepan, 2008), which needs to be elucidated with more follow-up samples over subsequent days to see if there was a subsequent increase in bone formation. Unfortunately long follow-up periods are usually difficult to obtain in free-living populations. The mechanical loading performed could have also overridden any effect of the pre-exercise feeding, therefore consuming the nutrients after exercise when the mechanical loading has already been performed, may be a better way to examine the effects of feeding on the bone turnover response. The response showed by Scott *et al.* (2012) may be specific to endurance running, as there are no studies that have compared different modes of exercise, with the same intensity, duration, or using the same bone turnover markers, there may be

sport or mode specific responses to exercise and feeding that need to be investigated (Banfi *et al.*, 2010; Rogers *et al.*, 2011).

De Souza *et al.* (2014) investigated the effects of CHO supplementation on markers of bone turnover in runners who performed an overload training programme for 8 days (13 sessions in total) and an intermittent high intensity running protocol (10 x 800m) on day 9, compared to a control group that consumed placebo solutions throughout. The CHO beverage consisted of 1 g·kg⁻¹·BM⁻¹ of maltodextrin per hour and was ingested during the running sessions on days 1 – 8 and a 7% maltodextrin solution was consumed during the intermittent running protocol on day 9 in the CHO group. Blood samples were taken on day 1, before the start of the 10 x 800m, immediately after the 10 x 800m and then after 80 minutes of recovery. In the control group, β -CTX concentrations increased from the start of the 10 x 800m to 80 minutes of recovery. The CHO beverage significantly reduced β -CTX compared to the control group. P1NP, OC and PTH increased significantly after the 10 x 800m in both groups. This suggests that CHO ingestion during high intensity, intermittent running, shifts bone turnover in a positive direction towards increased formation and decreased resorption. Although this investigation was not a cross-over design it is one of the first studies to report the impact of CHO supplementation during intensive training on bone turnover.

Finally, a study by Sale *et al.* (2015) compared the effect of feeding an 8% CHO drink (glucose) immediately before, every 20 minutes during and immediately after 120 minutes of running at 70% VO_{2max}, with consumption of a placebo drink at the same time points. The authors showed that ingestion of the CHO before, during and after exercise attenuated the β -CTX and P1NP responses 2 hours post-exercise compared to the placebo trial, but this response was not maintained during the 72 hours following exercise, where β -CTX and P1NP increased. Suppressing bone turnover needs to be considered alongside the requirements for bone adaptation to mechanical loading, as it may mean that the ability of the bone to adapt to the mechanical stimulus is blunted. Suppressed bone turnover is observed during bisphosphonate treatment which can lead to microdamage accumulation or unmineralised bone accumulation (Hirano *et al.*, 2000; Allen and Burr, 2011). This suppression of bone turnover may be detrimental in sub-elite populations where training is performed only once

per day or even less, and where the singular acute bouts may have an anabolic effect on bone. However, in elite endurance athletes training multiple times a day, the repetitive mechanical stimuli placed on the skeleton could have a more catabolic effect on bone (Nishiyama *et al.*, 1988; Hinton *et al.*, 2010; Oosthuysen *et al.*, 2013; Haakonssen *et al.*, 2014). This may mean that suppression of bone turnover or just bone resorption is only desired at some points of an athletic season or only after certain training sessions.

Further investigations are needed to establish the effect of suppressing post-exercise bone turnover on long-term bone remodelling and whether it translates to anabolic or catabolic bone adaptation in athletes. Sale *et al.* (2015) attribute the effects of the CHO drink to changes in IL-6, as ingestion of the CHO drink attenuated the rise in circulating IL-6, which is usually associated with exercise (Kwan *et al.*, 2004; Nieman *et al.*, 1998) and has been shown to activate osteoclastogenesis, osteoclast activity and bone resorption (Kotake *et al.*, 1996). Neither de Sousa *et al.* (2014) or Sale *et al.* (2015) investigated the effect of CHO ingestion during exercise on gastrointestinal discomfort, despite this commonly causing gastrointestinal symptoms in athletes (Rehrer *et al.*, 1992; Cox *et al.*, 2010; Pfeiffer *et al.*, 2012).

2.15 Summary

Stress fracture injuries are common and debilitating injuries amongst track and field athletes and endurance athletes, but the extent of these injuries amongst triathletes, is unknown. In addition, how the bones respond to the extreme training loads performed by triathletes and other endurance athletes necessitates further investigation. Triathletes often train three or four times a day, meaning that the amount of nutrients that they can consume around these sessions is limited. This often leads to poor fuelling and recovery and low energy availabilities, which are risk factors for stress fracture injuries.

Calcium homeostasis is an important mechanism in regulating the bone turnover response, and is controlled by PTH at rest. It has been hypothesised that high dermal calcium losses contribute to a

disruption in calcium homeostasis, but to date, the mechanism by which PTH is controlled during and after exercise has not been investigated.

Bone turnover can be measured via biochemical markers in the blood and urine. β -CTX and P1NP are the most reliable biochemical markers of bone turnover and can provide a cross-sectional view of bone turnover, which is particularly important for investigating the effects of exercise and feeding on bone turnover. Bone turnover markers are highly responsive to feeding and exercise; bone resorption increases with exercise and decreases with feeding at rest. Whilst there are studies that have investigated pre-exercise feeding and feeding during exercise, there are no studies that have investigated the effect of post-exercise feeding on bone turnover. This thesis reports four studies that extend the previous body of research into bone metabolism in endurance athletes.

CHAPTER 3: GENERAL METHODS

3.1 Participants

Male and female elite triathletes, male endurance trained runners and triathletes and male recreational runners volunteered for the studies in this thesis. All participants were informed of any risks associated with participation in the studies and experimental procedures were explained thoroughly to each participant. Participants were non-smokers and were not taking any medication or experiencing any condition known to affect bone metabolism. Compliance with these inclusion criteria was confirmed during the first visit to the laboratory or first encounter with the participants, where health screening (Appendix B) was also completed and written informed consent provided. All participants completed the ARTD Lifestyle questionnaire (Appendix C) which included recording any dietary supplementation. Participants were asked if supplementation changed at any point during participation in the studies and were asked to maintain normal training and activity levels. All studies were approved by the Ethical Advisory Committee at Nottingham Trent University.

3.2 Experimental protocols

3.2.1 Speed lactate and maximal oxygen uptake (VO_{2max}) test

In the studies reported in Chapters 6 and 7, participants performed an incremental test on a motorised treadmill (Pulsar, HP Cosmos, Germany) to determine their lactate threshold, followed by a ramp test to determine their VO_{2max} . The incremental test was continuous in nature and consisted of graded exercise steps of 3 minutes. Participants commenced running at 8 – 9 $km \cdot h^{-1}$ and the speed increased by 1 $km \cdot h^{-1}$ every 3 minutes thereafter. Participants had capillary blood samples taken via finger prick (Section 3.3.2.1) at the end of each incremental stage, to determine blood lactate concentrations (YSI 2300 Stat, YSI Incorporated, USA). The test was terminated when a significant change (over 1 $mmol \cdot L^{-1}$) in blood lactate compared to the previous stage measurement was recorded. After the incremental exercise test participants rested for 10 minutes before undertaking the ramp test. The ramp test was continuous in nature; with speed remaining constant and the gradient of the treadmill increasing by 1% every 1 minute until volitional exhaustion. During the test, participants wore a standard heart rate monitoring device (Polar Electro, Finland) strapped around their chest, which

provided a continuous recording of heart rate. Expired gas was continuously analysed and recorded by a ZAN 600 USB OXI breath-by-breath gas analyser (nSpire Health, Colorado, USA). These tests were used to determine the running velocities corresponding to 55%, 65% and 75% of $\text{VO}_{2\text{max}}$ during level running based on the regression of VO_2 and velocity.

3.3 Measurements

3.3.1 Height and body mass

Height and body mass were recorded during the first visits to the laboratory or first encounter with the participants in each study and pre- and post-exercise body mass was recorded in the studies reported in Chapters 6 and 7. Height was measured using a stadiometer (Seca GmbH, Hamburg, Germany) and body mass was recorded using digital scales (Seca GmbH, Hamburg, Germany) to the nearest 0.1 kg. Body mass was recorded with no shoes and minimal clothing.

3.3.2 Blood sampling

3.3.2.1 Finger prick capillary sampling

In the studies reported in Chapters 6 and 7, blood samples were obtained during the speed lactate tests, using the finger prick capillary technique. This involved cleaning the fingertip with an alcohol wipe and allowing to air dry before puncturing the skin with a spring propelled lancet (Unistik3, Owen Mumford, UK) and collecting the blood in a small heparin/EDTA coated tube. The participants' hands were warmed in a water bath prior to the first sample to ensure continuous blood flow when sampling. All needles, sharps, biological samples and contaminated materials were disposed of in appropriate bins.

3.3.2.2 Venepuncture

Blood samples were taken via venepuncture in the studies reported in Chapters 4, 5 and 7. Participants adopted a semi-recumbent position and rested for 5 – 10 minutes. A tourniquet was applied to the upper arm and a prominent forearm vein identified. The area was cleaned with an alcohol wipe and allowed to air dry before using a butterfly needle (Becton Dickinson, Valu-Set,

USA) to puncture the skin and draw blood. Between 10 – 20 mL of blood was drawn using syringes and subsequently dispensed into either pre-cooled K3E EDTA tubes (15%, 0.12 mL) (Becton Dickinson, Vacutainer System, USA), gently inverted 5 – 8 times and then centrifuged immediately at 3000 rev·min⁻¹, for 10 minutes at 5°C, to generate plasma, or into standard serum tubes (Becton Dickinson, Vacutainer System, USA), gently inverted 5 – 8 times, left to clot at room temperature for 60 minutes before being centrifuged at 2000 rev·min⁻¹, for 10 minutes at 5°C, to generate serum. Plasma and serum was subsequently transferred into 0.5 mL Eppendorf tubes and stored at -80°C until analysis.

3.3.2.3 Cannulation

Blood samples were taken via a cannula in the studies reported in Chapters 6 and 7. Participants adopted a semi-recumbent position and rested for 5 – 10 minutes. The same procedure as Section 3.3.2.2 was employed before inserting a cannula (Becton Dickinson, Venflon, USA). The cannula was secured and flushed with saline solution (0.9% Sodium Chloride) (Becton Dickinson, PosiFlush, SP Syringe, USA) between samples and at regular intervals. Between 5 – 20 mL of blood was drawn using syringes and subsequently dispensed as reported in Section 3.3.2.2.

3.3.3 Sweat collection

Sweat was collected in the studies reported in Chapters 4 and 5, during 4 training sessions that represented a range of the different training sessions performed by the participants; a long steady run (easy run; ER), a long steady cycle (easy bike; EB), a high-intensity run (hard run; HR) and a high-intensity cycle (hard bike; HB) (sweat collection was not possible during swimming sessions). Ambient conditions were recorded throughout these training sessions (Appendix D). Participants emptied their bladders and body mass was measured using digital electronic scales readable to 0.01 kg, whilst wearing minimal clothing. Empty drinks bottles were weighed using the same scales, then filled and weighed again, any food or gels that were taken (mainly during long rides) were also weighed. Participants were instructed to only drink the fluid provided and to retain empty food wrappers. Absorbent patches for sweat collection (Tegaderm +Pads, 3M, Loughborough, UK) were applied to three sites (forearm, chest and back). The skin was thoroughly cleaned with distilled, de-

ionised water and dried thoroughly with sterile gauze before the patches were applied. Participants collected any urine passed during sessions in a container, which was subsequently weighed. At the end of the training sessions, sweat patches were removed using sterile tweezers and immediately placed into sealed containers (Salivette, Sarstedt AG & Co, Germany). Participants towelled dry and were weighed again wearing the same clothing as the previous occasion. Drinks bottles and any food wrappers were weighed again. Sweat loss was calculated from the change in body mass after correction for the mass of ingested fluid/food and for any urine passed during the training session. Mass loss due to substrate exchange and to respiratory water loss was ignored, as this would have been a small component of the total mass loss. The sealed containers containing the sweat patches were subsequently centrifuged and the sample removed for the measurement of Ca^{2+} .

3.4 Blood analysis

3.4.1 Blood lactate

A YSI lactate analyser (YSI Life Sciences 2300 STAT Plus) was used to measure blood lactate during the incremental exercise test performed in Chapters 6 and 7. Blood was collected into small heparin/EDTA coated tubes via finger prick after each 3 minute stage, and was immediately sampled by the YSI. The YSI aspirates 25 μL of blood to obtain a measurement within 65 seconds. The measurement range for lactate is 0 – 30 $\text{mmol}\cdot\text{L}^{-1}$ and has a precision of 0.1 $\text{mmol}\cdot\text{L}^{-1}$.

3.4.2 Ca^{2+} and pH

Ca^{2+} and pH were measured in whole blood using a blood gas analyser (Radiometer ABL90 FLEX, Copenhagen, Denmark) during experimental trials reported in Chapters 6 and 7. After drawing, blood was immediately transferred to 65 μL capillary tubes and sampled by the blood gas analyser. Ca^{2+} is estimated directly between pH 7.2 – 7.6 with no pH correction applied. The inter- and intra-assay CV for Ca^{2+} was $\leq 3\%$ between 0.2 – 9.99 $\text{mmol}\cdot\text{L}^{-1}$ and for pH was $\leq 1\%$ between 4.000 – 11.000.

To confirm the manufacturer's reported CVs and stability of measurements made by the blood gas analyser, repeated measurements were briefly tested in our laboratory prior to the commencement of any studies reported in this thesis. Table 2 shows the CV of Ca²⁺ measured in 3 samples of blood over 4 minutes. Table 3 shows the CVs of Ca²⁺ and pH measured in 12 consecutive samples from the same participant at rest. The CVs measured in our laboratory were all smaller than those reported by the manufacturer, which were calculated using a minimum of 786 samples.

Table 2. CV for Ca²⁺ in repeated measurements of 3 blood samples.

Repeat measure	Sample 1 Ca²⁺ (mmol·L⁻¹)	Sample 2 Ca²⁺ (mmol·L⁻¹)	Sample 3 Ca²⁺ (mmol·L⁻¹)
1 min post blood draw	1.21	1.20	1.19
2 min post blood draw	1.21	1.19	1.21
3 min post blood draw	1.20	1.19	1.19
4 min post blood draw	1.20	1.19	1.19
Mean	1.21	1.19	1.20
SD	0.01	0.01	0.01
CV (%)	0.48	0.42	0.84

Table 3. CVs for Ca²⁺ and pH in 12 consecutive blood samples at rest.

Sample	Ca²⁺ (mmol·L⁻¹)	pH
1	1.28	7.368
2	1.29	7.377
3	1.26	7.375
4	1.27	7.393
5	1.27	7.386
6	1.27	7.392
7	1.26	7.389
8	1.27	7.388
9	1.26	7.383
10	1.25	7.400
11	1.24	7.399
12	1.26	7.417
Mean	1.27	7.39
SD	0.01	0.01
CV (%)	1.04	0.18

3.4.3 β -CTX, P1NP and PTH

β -CTX, P1NP and PTH were measured by ECLIA on a fully automated COBAS c501 system (Roche Diagnostics, Mannheim, Germany) in blood plasma, in the studies reported in Chapters 4, 5, 6 and 7 and were measured in singlet. The inter-assay CV for β -CTX was $\leq 3\%$ between 0.2 – 1.5 $\mu\text{g}\cdot\text{L}^{-1}$, with sensitivity of 0.01 $\mu\text{g}\cdot\text{L}^{-1}$. The inter-assay CV for P1NP was $\leq 3\%$ between 20 – 600 $\mu\text{g}\cdot\text{L}^{-1}$, with sensitivity of 8 $\mu\text{g}\cdot\text{L}^{-1}$. The inter-assay CV for PTH was $\leq 4\%$ between 1 – 30 $\text{pmol}\cdot\text{L}^{-1}$, with sensitivity of 0.8 $\text{pmol}\cdot\text{L}^{-1}$.

The β -CTX assay is specific for crosslinked isomerised type 1 collagen fragments, independent of the nature of the crosslink. The assay specificity is guaranteed through the use of two monoclonal antibodies each recognising linear β -8AA octapeptides and the assay therefore quantifies all type 1

collagen degradation fragments that contain the isomerised octapeptide β -8AA twice (β -CTX) (Christgau *et al.*, 1998; Rosenquist *et al.*, 1998). The β -CTX ECLIA uses a sandwich test principle involving two incubations. The P1NP assay detects both trimeric P1NP (derived from the trimeric collagen structure) and monomeric P1NP (the trimeric P1NP is broken down due to thermal degradation) (Jensen *et al.*, 1998; Brandt *et al.*, 1999). The P1NP assay measures total P1NP concentrations by a sandwich test principle involving two incubations. The intact PTH assay employs a sandwich test principle in which a biotinylated monoclonal antibody reacts with the N-terminal fragment (1-37) and a labelled monoclonal antibody reacts with the C-terminal fragment (38-84).

Analyses performed on the COBAS c501 system were carried out at the University of East Anglia (UEA) by either a trained laboratory technician or the principle investigator whilst visiting UEA.

3.4.4 Albumin, total calcium and PO₄

Albumin, total calcium and PO₄ were measured in serum with standard commercial assays supplied by Roche Diagnostics, performed on a fully automated COBAS c501 system (Roche Diagnostics, Mannheim, Germany) in the studies reported in Chapters 4 and 5. The inter- and intra-assay CV for total calcium was $\leq 2.5\%$ between 0.20 – 5.00 mmol·L⁻¹. The inter- and intra-assay CV for albumin was $\leq 1.3\%$ between 0.20 – 10.00 g·dL⁻¹. The inter- and intra-assay CV for PO₄ was $\leq 1.8\%$ between 0.10 – 6.46 mmol·L⁻¹. Albumin, total calcium and PO₄ were all measured photometrically and were measured in singlet.

Albumin, total calcium and PO₄ were measured using the ABX Pentra 400 (Horiba ABX, Montpellier, France) in the studies reported in Chapters 6 and 7. PO₄, total calcium and albumin were measured in serum, using standard colorimetric assays and spectrophotometric methods and were measured in duplicate. PO₄ was measured using phosphomolybdate, with an inter- and intra-assay CV of $\leq 3.6\%$ between 0.09 – 7.80 mmol·L⁻¹. Total calcium was measured using ortho-cresolphthalein complexone, with an inter- and intra-assay CV of $\leq 1.7\%$ between 0.04 – 5.00 mmol·L⁻¹. Albumin was measured using bromocresol green, with an inter- and intra-assay CV of $\leq 1.9\%$ between 0.02 – 5.99 g·dL⁻¹. Because fluctuations in protein, particularly albumin, may cause total

calcium levels to change independently of the Ca^{2+} concentration, total calcium concentrations were corrected to give an albumin-adjusted calcium (ACa) value: $0.8 \text{ mg}\cdot\text{dL}^{-1}$ was subtracted from the total calcium concentration for every $1.0 \text{ g}\cdot\text{dL}^{-1}$ by which the serum albumin concentration was greater than $4 \text{ g}\cdot\text{dL}^{-1}$ or $0.8 \text{ mg}\cdot\text{dL}^{-1}$ was added to the total calcium concentration for every $1.0 \text{ mg}\cdot\text{dL}^{-1}$ by which the serum albumin concentration was less than $4 \text{ mg}\cdot\text{dL}^{-1}$; $\left([\text{Albumin}] - 4\right) * -0.8 + [\text{Total Ca}]$.

Analyses performed on the COBAS c501 system were carried out at UEA by either a trained laboratory technician or the principle investigator whilst visiting UEA. Analyses performed on the ABX Pentra 400 were carried out at Nottingham Trent University by the principle investigator.

3.4.5 Vitamin D

Total 25(OH)D (the sum of the 25-hydroxy metabolites of D_2 and D_3) in serum was determined using a high-performance liquid chromatography–tandem mass spectrometer (Waters Acuity, Manchester, UK) as described by Owens *et al.* (2014). Measurements were performed in a laboratory meeting the performance target set by the Vitamin D External Quality Assessment Scheme Advisory Panel for 25(OH)D assays and the assay was validated against published acceptance criteria (Food and Drug Administration, 2001). Assay sensitivity was determined by the lower limit of quantification: $25(\text{OH})\text{D}_2 = 2.5 \text{ nmol}\cdot\text{L}^{-1}$ and $25(\text{OH})\text{D}_3 = 2.5 \text{ nmol}\cdot\text{L}^{-1}$. CVs for the assay were 10% across a working range of $2.5 - 625 \text{ nmol}\cdot\text{L}^{-1}$ for both $25(\text{OH})\text{D}_2$ and $25(\text{OH})\text{D}_3$. All vitamin D analyses were performed at UEA by a trained laboratory technician.

3.5 Sweat analysis

The sweat samples collected in the studies reported in Chapters 4 and 5 were analysed for calcium concentrations. Sweat samples (0.5 mL) were mixed thoroughly with an equal volume of calcium chloride of known concentration ($1 \text{ mmol}\cdot\text{L}^{-1}$) to ensure that the resulting samples had a calcium concentration above the detectable limit of $0.2 \text{ mmol}\cdot\text{L}^{-1}$. Ca^{2+} was subsequently analysed using a blood gas analyser (Radiometer ABL90 FLEX, Copenhagen, Denmark). The Ca^{2+} concentration of

the resulting sample was subtracted from the known calcium chloride concentration to give an estimated Ca^{2+} concentration of the sweat sample. The dermal calcium losses during training sessions were estimated from the measured Ca^{2+} concentrations and the estimated volume of sweat loss.

3.6 Dietary analysis

Nutritics Dietary Software (Nutritics Professional Nutrition Analysis Software, Dublin, Ireland) was used to assess habitual dietary intake for energy content, macronutrient content (percentages of carbohydrate, fat and protein), and micronutrient content in the studies reported in Chapters 4, 5, 6 and 7. Nutritics was also used to create specific, individualised diets that were followed during the lead in periods to experimental trials and on experimental trial days in the study reported in Chapter 7. The Nutritics database contains 258 different nutrients and over 125,000 foods. Where certain foods were not already in the database, they were added individually from packaging and internet information. Where portion sizes were not recorded on habitual diet diaries, demographic average portion sizes within the Nutritics database were used, which are available for over 5,500 foods.

3.7 Urine osmolality

Urine osmolality was measured using a handheld Osmometer (Osmocheck, Vitech Scientific, Horsham, UK) in the studies reported in Chapters 4, 5 and 7, which was used as an index of hydration status. Participants collected their first void of the day into a clean container. After using doubly distilled water to clean and calibrate the Osmometer, a small amount of urine was dropped onto the Osmometer using a pipette and was subsequently measured. Measurements were made in triplicate.

3.8 Statistical analysis

Statistical significance was accepted at an alpha level of $P \leq 0.05$. All statistical analyses were performed on raw data. Baseline concentrations were compared using one-way analysis of variance (ANOVA). Parametric assumptions of normality and sphericity were confirmed using the Shapiro-

Wilks test and Mauchly's test of Sphericity and where assumptions were violated, a transformation was applied to the data so that the assumptions were satisfied. Where parametric assumptions of normality and sphericity were violated and not satisfied after a transformation was applied to the data, a Mann-Whitney U test was performed. Data presented in Chapters 6 and 7 were subsequently analysed using a repeated measures ANOVA, with *Trial* and *Time* (of sampling) as within participant factors. Tukey's HSD *post-hoc* test was used to compare each time point against baseline and to compare trials at each time point, where appropriate. *Post-hoc* comparisons are reported with Cohen's *d* effect sizes, with $d=0.2$ considered as a small effect, $d=0.5$ considered as a medium effect and $d=0.8$ considered as a large effect (Cohen, 1988). Other statistical analyses were performed in Chapters 5 and 6. These statistical analyses were performed with Statistica (StatSoft, Tulsa, OK) and SPSS (IBM SPSS Statistics 22, Armonk, NY).

**CHAPTER 4: INVESTIGATING BONE
METABOLISM IN ELITE TRIATHLETES
DURING OFF-SEASON TRAINING**

4.1 Introduction

Triathletes have amongst the highest training volumes of all elite athletes. Due to the three disciplines involved; they will train between two and four times a day and only rest briefly when injured or during a tapering period prior to an important competition. This large volume of training (Oosthuysen *et al.*, 2013) combined with high intensity sessions (Scott *et al.*, 2010) and minimal rest days, creates ideal conditions to induce an increase in bone resorption and cause an imbalance in the bone turnover response, which has been implicated in the development of stress fracture injuries (Schaffler *et al.*, 1990). In addition, non-weight-bearing swimming and cycling followed by weight-bearing running, may further increase the risk for the development of stress fracture injury; the swim and bike may cause a hormonally mediated increase in bone remodelling (Primary Remodelling Hypothesis; Bennell *et al.*, 1996a), and the subsequent run may accelerate microdamage accumulation at sites of repetitive loading (Rector *et al.*, 2008) (Primary Microdamage Hypothesis; Bennell *et al.*, 1996a).

Bone health of triathletes is currently under-investigated. The cross training performed by triathletes may be more osteogenic compared to competing in a single discipline (Scofield and Hecht, 2012). Although this may be true in terms of higher BMD, which is likely to protect triathletes from osteoporosis in later life, the risk of stress fracture injury remains higher in triathletes compared to cyclists and swimmers, as triathletes also experience low-strain repetitive mechanical loading of the lower limbs during running, which cyclists and swimmers do not (Maïmoun *et al.*, 2004b; Scofield and Hecht, 2012). Anecdotally, triathletes suffer from a high number of stress fractures and some high profile athlete cases have been highlighted in the British media at critical phases of the triathlon season. Despite this, there is no published research into bone turnover and bone health in elite triathletes, which may be due to the limited availability to study this population.

Combining a triathlete's high volume, high intensity training regime with poor nutritional practices such as; inadequate fuelling and recovery with carbohydrate and protein, low calcium and vitamin D intakes and low energy availability, all of which are common in endurance athletes, considerably increases the risk of bone injuries (Bennell *et al.*, 1996b; Bennell *et al.*, 1999; Zanker and Swaine, 2000; Ihle and Loucks, 2004; Lappe *et al.*, 2008; Miller *et al.*, 2016). Elite endurance athletes expend

large amounts of energy due to the amount of training performed; Fudge *et al.* (2006) used the DLW method to calculate that elite Kenyan runners expended 3,492 kcal·d⁻¹ during normal training, however the athletes consumed 3,170 kcal·d⁻¹, resulting in a negative energy balance. Energy intakes should match energy expenditures to ensure weight maintenance and to reduce the chance of an athlete suffering from the symptoms of RED-S, over-training, injury and illness (Mountjoy *et al.*, 2014). However, in reality, this is often difficult for endurance athletes to attain and compensatory energy intakes are uncommon, particularly in short-term, high-volume training periods (Stubbs *et al.*, 2004; Drenowatz *et al.*, 2012).

The disruption of calcium homeostasis with exercise is a potential mediator of bone loss, which is based on the findings and the hypothesis that sweating during exercise can cause large dermal calcium losses, leading to an increase in PTH, which can subsequently cause an increase in bone resorption (Klesges *et al.*, 1996; Barry and Khort, 2007; Barry and Khort, 2008; Barry *et al.*, 2011). Scott *et al.* (2014) showed that exercise causes an increase in PTH concentrations at the end of exercise and a decrease in PTH concentrations below baseline levels during recovery from exercise. Large dermal calcium losses have been measured during cycling, so when cycling is followed by a prolonged running bout, encompassing low-strain repetitive loading, this may increase the chance of accelerated remodelling and microdamage accumulation. There are currently no studies that have investigated the difference in dermal calcium losses between cycling and running (Klesges *et al.*, 1996; Guillemant *et al.*, 2004; Barry and Khort, 2007; Martin *et al.*, 2007; Rector *et al.*, 2008; Barry *et al.*, 2011; Oosthuyse *et al.*, 2013). Klesges *et al.* (1996) showed a decrease in BMC over a basketball season and attributed this to high dermal calcium losses and insufficient calcium intakes to replace the dermal losses. The authors suggested that this may consequently cause disrupted bone turnover and amplified bone loss.

The aim of this study was to investigate what is influencing bone metabolism and bone health in elite British triathletes, by measuring resting bone turnover across a training week. Retrospective stress fracture injury occurrence throughout an athletes' lifetime, energy expenditures, nutritional practices and dermal calcium losses were also measured. This was done in an attempt to establish behaviours

that may contribute to the high risk of stress fracture injury in this athletic population and to identify possible areas for intervention and further study.

4.2 Methodology

4.2.1 Participants

16 elite triathletes (Table 4) participated in this study that was approved by the Ethical Advisory Committee at Nottingham Trent University (Application numbers 398, 409 and 413). Participants were Olympic distance triathletes on the British Triathlon World Class Performance Programme and were based at three different sites throughout the UK. There were 6 participants at site 1 (Leeds – 3 males and 3 females; participants 01 – 06), 8 participants at site 2 (Loughborough – 6 males and 2 females; participants 07 – 14) and 2 participants at site 3 (Stirling – 2 males; participants 15 – 16). The results for male and female triathletes are presented together throughout Chapters 4 and 5. Male and female triathletes have similar training schedules, perform similar training sessions and experience the same demands of the sport, so it is appropriate to make recommendations for triathletes as a whole group.

Table 4. Participant characteristics. Data are mean \pm 1SD.

	Males (n = 11)	Females (n = 5)
Age (y)	24 \pm 4	24 \pm 4
Height (m)	1.79 \pm 0.06	1.66 \pm 0.02
Body mass (kg)	68.8 \pm 4.9	59.5 \pm 2.3

4.2.2 Experimental design

In this cross-sectional study, participants performed their usual training load and maintained normal behaviours for the entirety of the testing period. Prior to testing, participants provided written informed consent and completed health, injury and menstrual status (females only) questionnaires (Appendix E). The testing period lasted 7 – 10 days and was completed during off-season training in November and December 2015. Fasted morning blood samples were taken between 06:30 and 07:30 prior to morning training sessions. Participants at sites 2 and 3 collected their first void for the measurement of urine osmolality. Dermal calcium losses were measured in cycling and running

training sessions in 10 participants (sites 2 and 3). Participants completed daily diet and training logs and coaches recorded training sessions using Training Peaks (<https://www.trainingpeaks.com/>) (Appendix F). Energy expenditure was measured using DLW in 5 participants (site 1).

4.2.3 Testing procedures

4.2.3.1 Morning blood and urine collection

On four (site 1) or five days (sites 2 and 3), participants arrived at the training facility between 06:30 and 07:00 following an overnight fast (from 22:00 the previous evening). Participants at sites 2 and 3 collected their first void for the measurement of osmolality (Section 3.7) and participants at site 1 collected their second void for the measurement of energy expenditure (Section 4.2.4). Participants adopted a semi-recumbent position on a bed and a blood sample (20 mL) was obtained via venepuncture from a prominent forearm vein (Section 3.3.2.2). Blood samples were taken at precisely the same time for each participant on each occasion.

4.2.3.2 Sweat collection

Sweat was collected from participants at sites 2 and 3 during four different training sessions (easy run, ER; easy bike, EB; hard run, HR; hard bike, HB) (Section 3.3.3).

4.2.3.4 Stress fracture history

History of stress fracture, stress response and impact fracture was assessed by a questionnaire (Appendix C; Questions 16 and 17). Participants recorded the age when the fracture/response occurred, the location and whether it was confirmed with a bone scan (MRI, CT or X-ray).

4.2.3.5 Dietary information

Diet logs were recorded for a minimum of 5 days (Appendix G). Participants used weighing scales and measuring cups to improve accuracy of recording. Recorded diet logs were analysed using

dietary analysis software (Nutritics Professional Nutrition Analysis Software, Dublin, Ireland) (Appendix H) (Section 3.6).

4.2.4 Energy expenditure by Doubly Labelled Water

4.2.4.1 Isotope dosing and sampling

Five participants from site 1 provided baseline urine samples before receiving a weighed oral dose of $^2\text{H}_2^{18}\text{O}$ (day 0). The dose was equivalent to $70 \text{ mg}\cdot\text{kgBM}^{-1}$ deuterium oxide (DLM-2259, Deuterium Oxide 99.8At%, Sterility Tested, Goss Scientific Ltd., Nantwich, Cheshire, UK) and $200 \text{ mg}\cdot\text{kgBM}^{-1}$ of H_2^{18}O (Taiyo Nippon Sanso Water-18O Normalized 10 atom% ^{18}O , Sercon Ltd., Crewe, UK). Post-dose urine samples; the second void of the day, were collected daily for 10 days and the time of day noted. Urine samples were stored at -20°C until analysis in singlet using isotope-ratio mass spectrometry (IRMS).

4.2.4.2 Isotopic analyses

Measurements of $^2\text{H}/^1\text{H}$ ratios were made by dual inlet IRMS (Isoprime, GV Instruments Ltd, Wythenshawe, Manchester, UK). Samples of 0.4 mL were placed in 3.7 mL glass bottles with rubber septa (50 x 12.5 mm, non-evacuated vials, Labco Ltd, Lampeter, UK) flush filled with hydrogen (Gilson GX271 Autosampler, a1envirosciences, Düsseldorf, Germany) in the presence of a platinum catalyst and equilibrated at 22°C for 6 hours. All measurements were corrected for interference of H_3^+ ion in the ratio measurements and made relative to laboratory standards calibrated with values of -49.48, 182.05, 413.58, 876.64 ‰ relative to Vienna-standard mean ocean water (V-SMOW) (International Atomic Energy Agency, Vienna, Austria), before being expressed relative to V-SMOW. Measurements of $^{18}\text{O}/^{16}\text{O}$ ratios were made using continuous flow IRMS (AP2003, Analytical Precision Ltd, Northwich, Cheshire, UK). Samples of 0.5 mL were placed in 12 mL vacutainers (Labco Ltd, Lampeter, UK), flush filled with 5% CO_2 in nitrogen and then equilibrated by rotating overnight at room temperature. Sample enrichments were expressed relative to V-SMOW, first being made relative to laboratory standards calibrated with values of -6.99, 45.90, 98.79, 204.56 ‰ relative to V-SMOW.

4.2.4.3 Energy expenditure calculations

Total energy expenditure (TEE) was calculated as described by Schoeller *et al.* (1986) from slopes and intercepts of the isotope disappearance curves. Respiratory quotient was assumed to have a value of 0.85, based on the consumption of a standard Western diet (Ainslie *et al.*, 2003). Resting metabolic rate (RMR) was estimated using the Schofield equations (1985). Activity energy expenditure was calculated as $(TEE \times 0.9) - RMR$, assuming that diet-induced thermogenesis was 10% of TEE (Plasqui *et al.*, 2005). Physical activity level was expressed using TEE as a multiple of RMR (Ekelund *et al.*, 2001).

4.2.5 Blood sample analysis

Blood was treated and stored according to Section 3.3.2.2. β -CTX, P1NP and PTH were measured by ECLIA on a fully automated COBAS c501 system (Roche Diagnostics, Mannheim, Germany) (Section 3.4.3). Albumin, total calcium and PO_4 were measured with standard commercial assays supplied by Roche Diagnostics performed on the COBAS c501 system (Section 3.4.4) and total 25(OH)D (the sum of the 25-hydroxy metabolites of D_2 and D_3) in serum was measured using a high-performance liquid chromatography–tandem mass spectrometer (Section 3.4.5).

4.2.6 Sweat analysis

Sweat was analysed for calcium concentrations and the dermal calcium losses during training sessions were estimated from the measured calcium concentrations and the estimated volume of sweat loss (Section 3.5).

4.2.7 Bone turnover markers and metabolites

Due to there being no reference ranges for resting bone turnover markers and related metabolites available for elite athletes, the results in the present study were compared to resting concentrations for recreationally active individuals (RA) that have been collected by the Musculoskeletal Physiology Research group at Nottingham Trent University (Appendix A). Average concentrations,

+1 SD and -1 SD were plotted on each graph for comparison and are referred to as 'RA' concentrations throughout Sections 4.3 and 4.4. To give a numerical value to the difference between bone formation and bone resorption, the bone turnover marker ratio was calculated using the following equation: $[P1NP]/([\beta\text{-CTX}]*100)$, with a value above 1 indicating that bone turnover favours bone formation and a value below 1 indicating that bone turnover favours bone resorption. This equation was used in Lombardi *et al.* (2012) but for different bone turnover markers.

4.3 Results

4.3.1 Stress fracture history

Participants self-reported that all stress fractures were confirmed by a bone scan (MRI, CT or X-ray). There were 15 incidences of stress fracture injury amongst the 16 triathletes throughout their lifetimes. Nine triathletes had suffered 1 stress fracture and 3 triathletes had suffered 2 stress fractures.

Table 5. Stress fracture history of participants. The first stress fracture injury occurred at an average age of 22 ± 3 years, with a range of 19 to 27 years.

Fracture history	Males (n = 11)	Females (n = 5)
Stress fracture incidence	9	6
Multiple stress fracture incidence	1	2
Stress response incidence	3	0
Impact fracture incidence	8	3
Percentage of athletes that have suffered a stress fracture	73%	80%
Total percentage of athletes that have suffered a stress fracture	75%	

Participant	Stress fracture incidence	Stress fracture site(s)
01	1	Femur
02	1	Femur
03	1	Third metatarsal
04	2	Both navicular
05	1	Fibula
06	2	Navicular and 4 th metatarsal
07	0	N/A
08	1	Sacrum
09	1	Tibia
10	1	Sacrum
11	1	Tibia
12	1	Navicular
13	0	N/A
14	0	N/A
15	2	Metatarsal and tibia
16	0	N/A

4.3.2 Menstrual status

Three of the female participants were using combined oral contraception (2 Levest and 1 Loestrin 20) and one participant was using an implant. One participant was not using any hormonal contraception and had a regular menstrual cycle, which was between 28 and 35 days. Prior to using hormonal contraception, two of the participants had suffered from secondary amenorrhea.

4.3.3 Bone turnover markers

4.3.3.1 β -CTX

β -CTX concentrations were higher than average (RA) concentrations in 13 out of 16 participants in all samples and did not increase or decrease in any sample. The minimum concentration was 0.38 ng·mL⁻¹ and the maximum concentration was 1.76 ng·mL⁻¹. Participant 13 (site 2) had average β -CTX concentrations that were more than 3 times the average (RA) concentration (Figure 7A).

4.3.3.2 P1NP

P1NP concentrations were higher than average (RA) concentrations in 13 out of 16 participants in all samples and did not increase or decrease in any sample. The minimum concentration was 42.5 ng·mL⁻¹ and the maximum concentration was 178.0 ng·mL⁻¹. Participants 13 (site 2) and 15 (site 3) had average P1NP concentrations that were more than 2 times the average (RA) concentration (Figure 7B).

4.3.3.3 Bone turnover marker ratios

Table 6. Average bone turnover marker ratios.

Participant	Bone turnover marker ratio
01	1.40
02	1.37
03	1.23
04	1.66
05	1.28
06	1.15
07	1.23
08	1.44
09	0.82
10	1.11
11	1.04
12	1.07
13	1.04
14	1.17
15	1.56
16	1.36

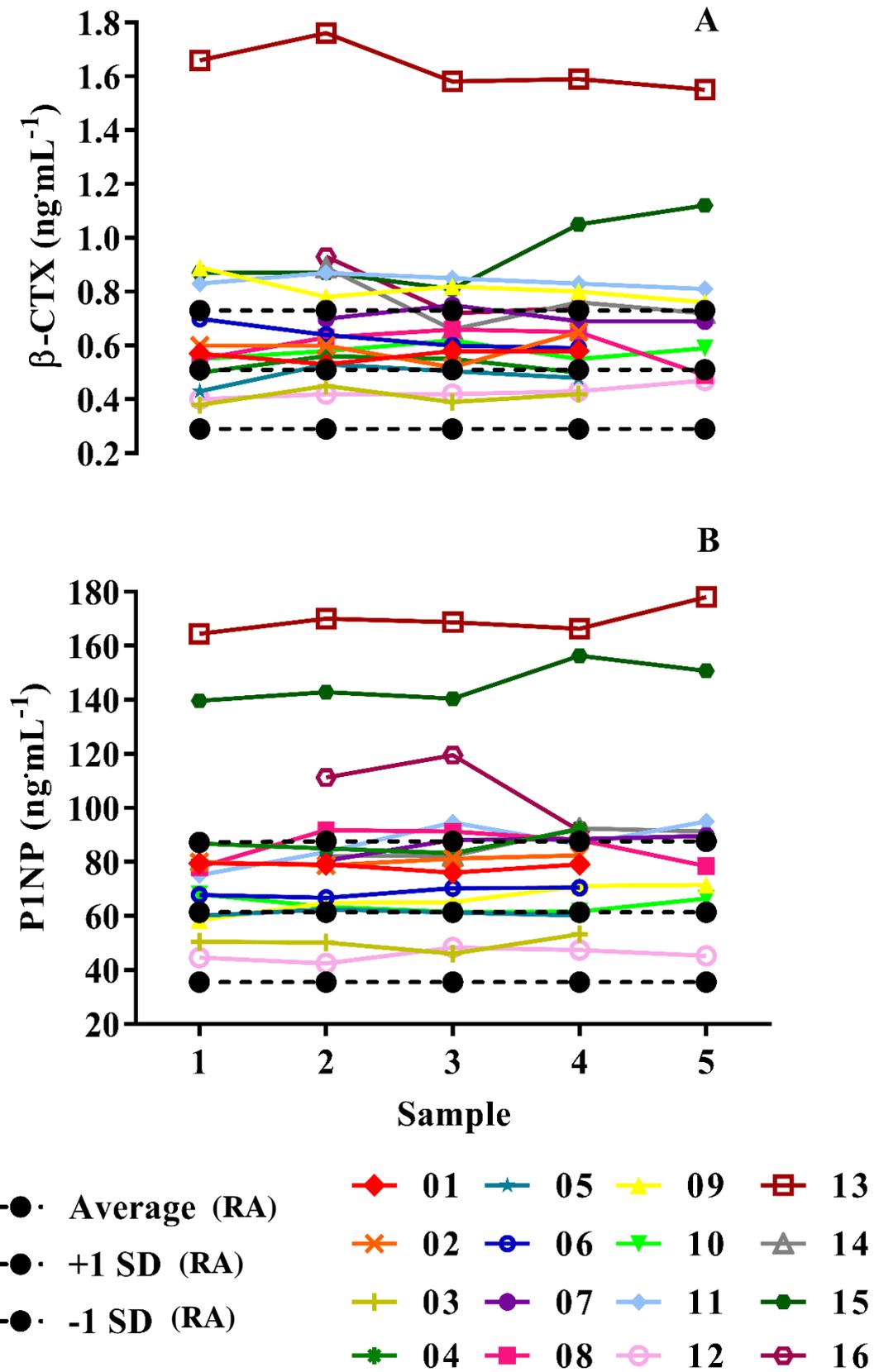


Figure 7. Resting concentrations of β -CTX (A) and P1NP (B) for all participants in all blood samples over 7 – 10 days during off-season training.

4.3.4 Calcium metabolism

4.3.4.1 PTH

PTH concentrations were lower than average (RA) concentrations in 9 out of 16 participants in all samples. Concentrations increased in sample 3 in 4 out of 6 participants from site 1 (sample 3 is missing from participant 05 due to injury), which was the morning after a hard/long training day. The minimum concentration was $1.8 \text{ pmol}\cdot\text{L}^{-1}$ and the maximum concentration was $5.4 \text{ pmol}\cdot\text{L}^{-1}$. The PTH concentration in sample 4 was almost 2 times the average (RA) concentration in participant 04 (site 1). The PTH concentration in samples 1 and 2 was almost 1.5 times the average (RA) concentration in participant 05 (site 1) (Figure 8A).

4.3.4.2 ACa

ACa concentrations were lower than average (RA) concentrations in 13 out of 16 participants in all samples. ACa concentrations increased in sample 3 for participants 01 and 16 (site 1 and 3) and decreased in sample 3 for participant 04 (site 1), which coincided with an increased PTH concentration. The minimum concentration was $2.21 \text{ mmol}\cdot\text{L}^{-1}$ and the maximum concentration was $2.49 \text{ mmol}\cdot\text{L}^{-1}$ (Figure 8B).

4.3.4.3 PO₄

PO₄ concentrations were higher than average (RA) concentrations in 7 out of 16 participants in all samples. Concentrations increased in sample 3 in participants 01 and 02 (site 1) and in sample 4 in participant 09 (site 2). The minimum concentration was $0.82 \text{ mmol}\cdot\text{L}^{-1}$ and the maximum concentration was $1.66 \text{ mmol}\cdot\text{L}^{-1}$. The PO₄ concentration in sample 1 was almost 1.5 times the average (RA) concentration in participant 03 (site 1) (Figure 8C).

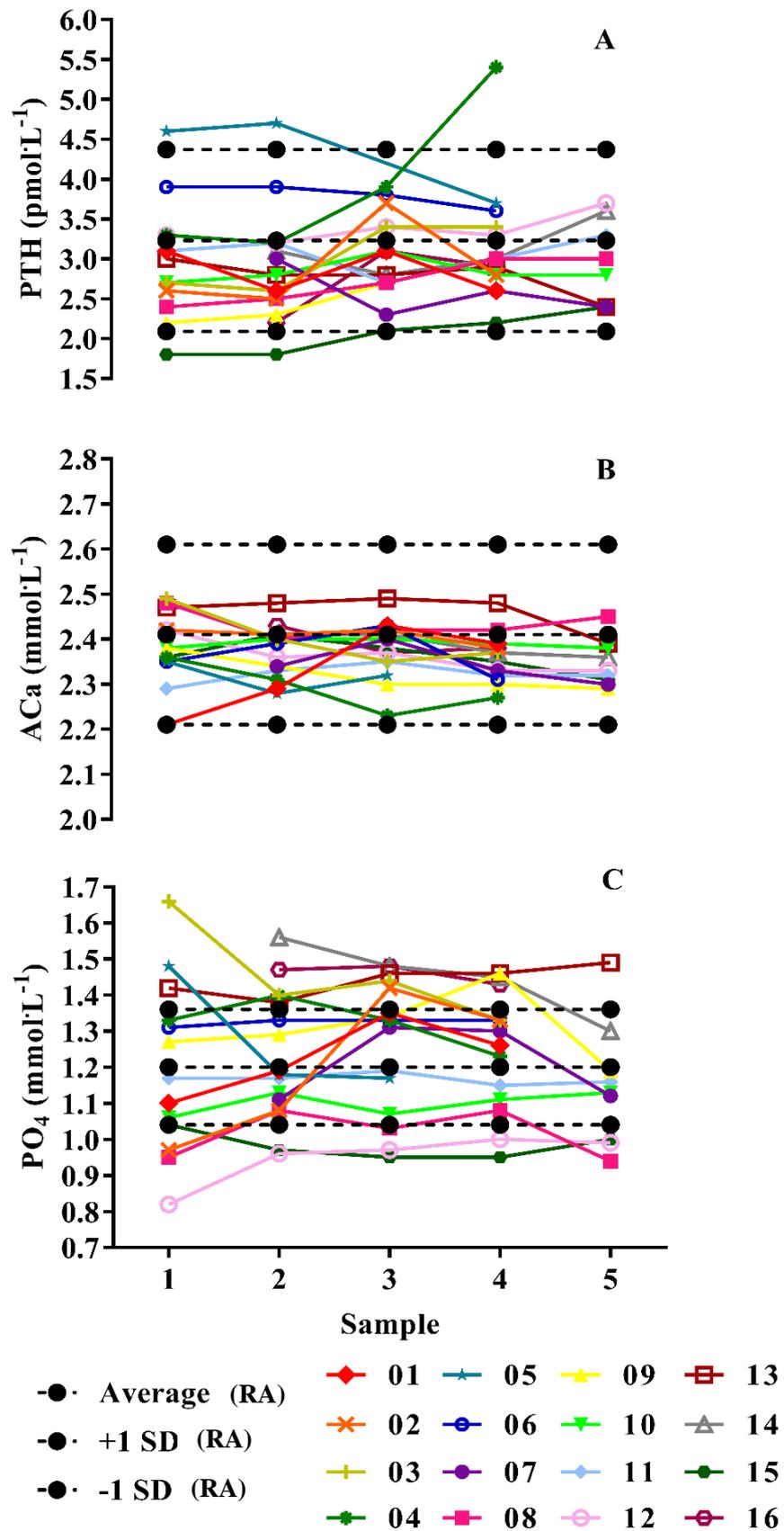


Figure 8. Resting concentrations of PTH (A), A Ca (B) and PO₄ (C) for all participants in all blood samples over 7 – 10 days during off-season training.

4.3.6 Total energy expenditure

Table 7. Average daily energy expenditure for participants 01 – 05 (site 1) during 10 days of off-season training, as measured by DLW.

Participant	Age (y)	Sex	Body mass (kg)	Height (m)	Average daily energy expenditure (kcal)
01	25	M	69.8	1.78	5,877
02	27	M	73.0	1.85	5,906
03	24	M	64.0	1.71	5,506
04	26	F	63.0	1.69	4,381
05	29	F	59.0	1.68	2,877 (sustained injury on day 2)
Mean ± SD	26 ± 2		65.8 ± 5.6	1.74 ± 0.07	4,909 ± 1,294

4.3.7 Energy intakes and hydration status

Table 8. Average daily energy, CHO, PRO and calcium intakes and average urine osmolality for all participants during off-season training. Data are averaged from a minimum of 5 day diet logs and 5 urine samples. Diet logs are missing for participants 02 and 03. The first void was not collected for participants at site 1. Participants were classed as hydrated at ≤ 500 mOsmL⁻¹, dehydrated at 501 – 999 mOsmL⁻¹ and severely dehydrated at ≥ 1000 mOsmL⁻¹.

Participant	Sex	Body mass (kg)	Average	Average	Average	Average	Average	Average	Average
			daily energy intake (kcal)	daily carbohydrate intake (g)	daily carbohydrate intake (% of total intake)	daily protein intake (g)	daily protein intake (% of total intake)	daily calcium intake (mg)	daily urine osmolality (mOsmL ⁻¹)
01	M	69.8	3,198	499	62	126	16	1,321	N/A
02	M	73.0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
03	M	64.0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
04	F	63.0	3,540	457	52	153	17	1,665	N/A
05 (injured)	F	59.0	3,381	441	52	138	16	2,428	N/A
06	F	57.0	3,448	426	49	112	13	1,302	N/A
07 (injured)	F	60.4	1,904	231	49	88	18	967	875
08	M	71.5	2,524	276	44	127	20	1,352	826
09	M	70.7	2,378	265	45	127	21	1,451	968
10	M	73.0	3,231	363	45	144	18	1,480	880
11	M	65.0	2,235	296	53	84	15	748	974
12	F	58.0	2,709	347	51	109	16	1,220	454
13	M	73.5	2,417	336	56	99	16	1,036	970
14	M	66.5	2,176	276	51	100	18	978	944
15	M	58.0	3,020	634	84	76	10	1,029	480
16	M	72.0	2,893	418	58	137	19	1,987	910
Mean ± SD		65.9 ± 6.1	2,790±528	376 ± 111	54 ± 10	116 ± 24	17 ± 3	1,355±444	828 ± 196

4.3.8 Supplementation

Table 9. Supplements consumed for all participants during off-season training.

Participant	Supplementation
01	Vitamin D and calcium
02	Vitamin D, calcium, multivitamin, vitamin B12, omega 3
03	Multivitamin and vitamin B12
04	Vitamin D and omega 3
05	Vitamin D
06	Vitamin D and calcium
07	Vitamin D
08	Vitamin D and calcium
09	None
10	Vitamin D and calcium
11	Vitamin D, calcium and omega 3
12	None
13	Vitamin D, calcium and multivitamin
14	None
15	Vitamin D and iron
16	Iron

Vitamin D = 1,000 IU (25 µg) vitamin D₃; calcium = 267 mg; multivitamin contained 5 µg of vitamin D₃ and 200 mg of calcium; omega 3 = 200 mg DHA and 300 mg EPA; vitamin B12 = 500 µg; iron = 14 mg.

4.3.9 Training loads

Table 10. Total training time, minutes of swimming, cycling, running and strength and conditioning (S&C) sessions for all participants during 7 days of off-season training.

Participant	Total swim time over 7 days (mins)	Total cycling time over 7 days (mins)	Total run time over 7 days (mins)	S&C over 7 days (mins)	Total over 7 days (mins)
01	390	1,085	430	90	1,995
02	390	1,170	245	90	1,895
03	530	950	200	90	1,770
04	465	620	255	90	1,430
05 (injured)	225	210	60	45	540
06	390	570	60	45	1,065
07 (injured)	240	420	0	180	840
08	525	660	470	225	1,880
09	405	720	455	225	1,805
10	405	780	425	225	1,835
11	405	780	425	225	1,835
12	405	720	440	225	1,790
13	405	740	400	225	1,770
14	405	740	400	225	1,770
15	497	730	384	110	1,721
16	400	525	355	105	1,385
Mean ± SD	405 ± 83	714 ± 234	313 ± 157	151 ± 73	1,583 ± 422

4.3.10 Sweat losses and dermal calcium losses

Table 11. Total sweat and calcium losses and rates of sweat and calcium losses for participants at sites 2 and 3. Easy run (ER), easy bike (EB), hard run (HR) and hard bike (HB). The average concentration of calcium in sweat was $0.54 \pm 0.09 \text{ mmol}\cdot\text{L}^{-1}$. Data are mean \pm 1SD.

	Training session			
	ER	EB	HR	HB
Total sweat loss (L)	0.77 ± 0.30	1.09 ± 0.31	0.50 ± 0.38	0.87 ± 0.31
Total calcium loss (mg)	75 ± 25	108 ± 35	51 ± 34	85 ± 32
Rate of sweat loss (L·h ⁻¹)	0.67 ± 0.23	0.43 ± 0.16	0.76 ± 0.51	0.51 ± 0.22
Rate of calcium loss (mg·h ⁻¹)	68 ± 34	43 ± 21	82 ± 59	48 ± 18

4.4 Discussion

The main findings of the study are: 1) concentrations of both β -CTX and P1NP were higher than average (RA) concentrations in 13 out of 16 participants; 2) PTH concentrations were consistently lower than average (RA) concentrations in 9 out of 16 participants, but concentrations tended to increase the morning after hard/long training days; 3) ACa concentrations were consistently lower than average (RA) concentrations in 13 out of 16 participants; 4) PO_4 concentrations were higher than average (RA) concentrations in 7 out of 16 participants; 5) participants 01 – 05 expended large amounts of energy and did not have sufficient energy intakes to match; 6) dermal calcium losses were high during running and cycling sessions and hard running sessions induced the highest rates of calcium and sweat loss.

This is the first study that has investigated bone metabolism in elite triathletes. Few studies have investigated the prevalence of overuse injuries in elite or sub-elite triathletes (Vleck and Garbutt, 1998; Duckham *et al.*, 2012) and to our knowledge only one study has measured bone turnover markers at the start and end of a triathlon season in sub-elite triathletes (Maimoun *et al.*, 2004a). Consequently, there are no reference ranges for bone turnover markers and related metabolites available for triathletes or other elite athletes (Lombardi *et al.*, 2011). Further, due to this group of triathletes not having a rest day from training, it was impossible to obtain a baseline sample for each participant, therefore the results of the present study are compared to resting concentrations of recreationally active individuals that have been collected by the Musculoskeletal Physiology Research group at Nottingham Trent University during numerous studies (Appendix A). However, this comparison should be interpreted with caution, as the data from the recreationally active individuals are from resting blood samples, where the individuals refrained from exercise for a minimum of 24 hours prior to the blood sample, whereas, the triathletes' blood samples were taken after consecutive days of exercise.

We have shown that recreationally active individuals have mean resting bone turnover marker concentrations that are higher than the non-active, healthy population (Appendix A). The average concentrations of β -CTX ($0.69 \pm 0.29 \text{ ng}\cdot\text{mL}^{-1}$) and P1NP ($81.14 \pm 33.31 \text{ ng}\cdot\text{mL}^{-1}$) in the present

study were high compared to the non-active population (β -CTX; 0.23 – 0.32 ng·mL⁻¹ and P1NP; 31.00 – 49.40 ng·mL⁻¹) and recreationally active individuals (β -CTX; 0.51 ± 0.22 ng·mL⁻¹ and P1NP; 61.40 ± 25.89 ng·mL⁻¹). The fact that concentrations of both bone turnover markers are high suggests that overall bone turnover is elevated compared to non-active and recreationally active individuals. This may lead to accelerated microdamage accumulation (Martin, 1992; Bennell *et al.*, 1996a), increased bone fragility (Burr *et al.*, 1997), increased trabecular resorption depth, increased intracortical porosity and decreased stiffness of the bone (Eriksen *et al.*, 1999), all of which will increase the risk of stress fracture injury (Parfitt, 1982; Schaffler *et al.*, 1990; Burr, 2002; Schaffler, 2003; Parfitt, 2004).

It should be noted that the majority of the triathletes had urine osmolality measures that reflected a dehydrated state at the time of blood sampling; this could mean that haemoconcentration produced artificially high concentrations of bone turnover markers and metabolites. Participants 12 and 15 were the only triathletes that were in a hydrated state. Participant 12 had the lowest bone turnover marker concentrations, but participant 15 had amongst the highest concentrations, suggesting that plasma volume did not influence bone turnover marker concentrations.

Participant 13 had β -CTX and P1NP concentrations that were 2 – 3 times higher than average (RA) concentrations, with a peak β -CTX concentration of 1.76 ng·mL⁻¹ and a peak P1NP concentration of 178.00 ng·mL⁻¹. This participant was relatively young (20 years old) and may still have been in the later stages of growth (Tanner, 1975), where high bone turnover is present due to bone modelling, however, this subsides as bone growth slows and the newly formed osteoid undergoes mineralisation (Weaver *et al.*, 1997; Weaver, 2000; Bachrach, 2001; Eastell *et al.*, 2005). Therefore, these high concentrations may require further investigation.

Participant 13 also had one of the lower bone turnover marker ratios (1.04) compared to other participants. Only participant 9 had a bone turnover marker ratio below 1, suggesting that for all other participants, bone turnover favours bone formation rather than bone resorption. However, the accelerated bone turnover that is present in these athletes may override the positive effect of this

bone turnover balance. Bone remodelling may be occurring too quickly for the processes of osteoclastic bone resorption, reversal and osteoblastic bone formation to be completed properly. Although the amount of bone formation may exceed the amount of bone resorption at rest, if the osteoblasts do not entirely fill the resorption pits created on the bones surface (Salo *et al.*, 1997; Teitelbaum and Ross, 2003; Stenbeck and Horton, 2004) and the osteoid is not yet mineralised before another training session is performed, which induces an acute increase in bone resorption (Scott *et al.*, 2010), osteoclasts may resorb the same newly deposited bone. This may create localised weakened areas on the bones surface where microdamage accumulation is more likely to occur (Martin, 1992; Bennell *et al.*, 1996a; Eriksen *et al.*, 1999). Considering that complete new bone mineralisation can take up to 4 months (Stewart and Hannon, 2000; Hadjidakis and Androulakis, 2006; Crockett *et al.*, 2011), there could be numerous weakened areas on an athlete's bone.

The higher than average (RA) bone turnover marker concentrations are in agreement with the findings of Waldron-Lynch *et al.* (2010), who showed that professional jockeys had NTX and PINP concentrations that were more than 2 times higher than control participants. The authors attribute the high bone turnover to low calcium intakes and energy deficiencies, with average energy and calcium intakes of $1,760 \pm 283 \text{ kcal}\cdot\text{d}^{-1}$ and $541 \pm 106 \text{ mg}\cdot\text{d}^{-1}$. In the present study β -CTX and PINP concentrations were higher than average (RA) in all samples in 13 out of 16 participants; there are no increases and decreases on certain mornings. This suggests that either the effects of training sessions performed did not persist to the following morning, or that all training sessions had the effect of elevating bone turnover the following mornings. Oosthuysen *et al.* (2013) showed that β -CTX and BALP concentrations remained elevated the mornings after 3 h of cycling on 4 consecutive days. Without a true baseline sample in the present study, this is difficult to interpret. With different training schedules each day it may be expected for bone turnover to be more elevated on certain mornings than others, due to different mechanical loading and different hormonal responses to different training sessions.

One of the reasons for the high concentrations of bone turnover markers could be the high amounts of energy expended on a daily basis. Hinton *et al.* (2010) and Rehrer *et al.* (2010) showed that CTX

concentrations increased mid-way through a 6 day cycling tour, which coincided with decreased energy intakes. Westerterp *et al.* (1986) measured energy expenditure in 4 cyclists during the Tour de France and showed that the average daily energy expenditure was 8,054 kcal·d⁻¹ when covering 3,500 km over 23 days. In contrast, Schulz *et al.* (1992) and Edwards *et al.* (1993) calculated that 9 female distance runners expended only 2,768 kcal·d⁻¹ and 2,990 kcal·d⁻¹. All of these studies measured energy expenditure by DLW. The 3 male participants in the present study expended between 5,506 and 5,906 kcal·d⁻¹ and the female participant that was not injured expended 4,381 kcal·d⁻¹. These energy expenditures are high in comparison to athletes of other sports, other than cyclists competing in cycling tours (Westerterp *et al.*, 1986; Rherer *et al.*, 2010), which are extreme endurance events. As the phase of training changes, volumes and intensities of training sessions will change, and with the addition of races and training camps, it is likely that energy expenditures will change accordingly. This group of triathletes often spend time training at altitude, which itself increases energy expenditure due to increases in RMR and diet-induced energy expenditure (Pulfrey and Jones, 1996; Reynolds *et al.*, 1999; Westerterp-Plantenga *et al.*, 1999), therefore energy expenditures are likely to exceed the values reported here at other phases of the season.

Participants did not match energy intakes with energy expenditures, meaning that they are likely to be in a negative energy balance, and this may contribute to the high bone turnover. As we only measured energy expenditure in 5 participants, we can only assume that as the total volume of training is similar between participants, it is likely that the male triathletes would expend between 5,000 and 6,500 kcal·d⁻¹ and the females between 4,000 and 5,000 kcal·d⁻¹ during off-season training. It should be noted that this can vary drastically between individuals due to differences in RMR (Harris and Benedict, 1918; Thompson and Manore, 1996; Speakman *et al.*, 2004), which means that these guideline energy expenditures may not be relevant to every triathlete.

A negative energy balance is detrimental for bone health and can cause symptoms of RED-S or the triad (Nattiv *et al.*, 2007; De Souza *et al.*, 2014; Mountjoy *et al.*, 2014). Inadequate energy intakes are common in endurance athletes (Edwards *et al.*, 1993; Thompson *et al.*, 1995; Burke *et al.*, 2003; Melin *et al.*, 2015), and athletes have regularly been shown to have low energy availabilities (Fudge

et al., 2006; Loucks, 2007; Drenowatz *et al.*, 2012; VanHeest *et al.*, 2014). Average energy intakes reported in the present study are $2,675 \pm 414 \text{ kcal}\cdot\text{d}^{-1}$ for the male triathletes and $3,070 \pm 780 \text{ kcal}\cdot\text{d}^{-1}$ for the female triathletes. These low energy intakes could be occurring due to the appetite suppressing effect of endurance training (King *et al.*, 1994; King *et al.*, 1997; Hubert *et al.*, 1998) and the lack of a strong biological drive to match energy intakes with energy expenditures, or due to purposeful restriction with the aim of reducing body weight, which is common in endurance and aesthetic sports (Truswell, 2001; Nattiv *et al.*, 2007). Perhaps a more likely reason for inadequate energy intakes in this group of triathletes is the lack of time available to consume food in between training sessions. The (non-injured) triathletes trained on average for 4 – 5 hours each day during this training phase, with sessions being spread out throughout the day, meaning that the time that they have to consume food and digest it prior to the next training session is limited, as eating prior to training can cause gastrointestinal discomfort (Rehrer *et al.*, 1992; Cox *et al.*, 2010; Pfeiffer *et al.*, 2012). Therefore, consuming the necessary energy in large meals throughout the day is not practical and other feeding practices that allow the consumption of appropriate energy and macronutrients in between training sessions need to be explored.

Under reporting of diet logs may have occurred in the present study, which is one of the limitations of using self-report to measure energy intakes (Schoeller, 1995; MacDiarmid and Blundell, 1998; Ebine *et al.*, 2002; Burke *et al.*, 2005; Drenowatz *et al.*, 2012). The lower intakes reported by the males compared to the females may be due to greater underreporting by the males. For example, according to the self-reported diet logs of participant 01, this male triathlete has an energy balance of $-2,679 \text{ kcal}\cdot\text{d}^{-1}$. This would have extreme negative health and performance implications if this was maintained on a daily basis and would also cause drastic weight loss, which did not occur throughout the study period, suggesting that $3,198 \text{ kcal}\cdot\text{d}^{-1}$ was not the full energy intake of participant 01. Similarly, Silva *et al.* (2012) showed that elite basketball players under reported dietary intakes by 38% and Trappe *et al.* (1997) showed an energy availability of $-2,457 \text{ kcal}\cdot\text{d}^{-1}$ in female swimmers, despite stable body weights. It is difficult to accurately assess energy intakes amongst participants in a field setting as methods generally rely on self-report and participant motivation to record food

intake in sufficient detail. This is discordant with the DLW method of measuring energy expenditure, meaning that it is difficult to accurately measure energy balance.

Amenorrhea is one of the entities of the triad and 2 of the female participants from site 1 had previously suffered from secondary amenorrhea, prior to hormonal contraceptive use, which is often caused by low energy availability (Nattiv *et al.*, 2007; De Souza *et al.*, 2014; Mountjoy *et al.*, 2014). Hormonal contraceptive use can mask amenorrhea, due to the decrease in oestradiol which inhibits ovulation (Baird and Glasier, 1993; Grinspoon *et al.*, 2003). Despite differences in oestradiol concentrations between the female participants, they have a similar bone turnover response to the training performed, which may suggest that the mechanical loading experienced during triathlon training could override the effect of reproductive hormones on bone metabolism. There has been acknowledgement of similar reproductive hormone deficiencies occurring in male athletes (Hackney, 2008; Tenforde *et al.*, 2015), which has contributed to the development of RED-S. Due to RED-S being a recent development (Mountjoy *et al.*, 2014), evidence for the symptoms for RED-S occurring in male athletes is limited. This is one of the first studies that has shown that elite male endurance athletes have both high bone turnover and low energy intakes, that are likely to mean the athletes have low energy availabilities.

As well as overall energy intake, macronutrient composition of the diet is also important for maintaining normal bone turnover. A low CHO intake has been shown to cause increased osteoclast activity and poor bone mineralisation, leading to decreases in BMD and bone quality (Hou *et al.*, 1990; Li *et al.*, 1990; Zernicke *et al.*, 1995; Wohl *et al.*, 1998; Kettler, 2001; Bielohuby *et al.*, 2010). Low CHO diets may be deficient in calcium (Freedman *et al.*, 2001) and cause metabolic acidosis that promotes calcium mobilisation from the bone (Barzel and Massey, 1998; Lemann, 1998). Low CHO diets may also increase stress hormone and cytokine responses to exercise, including adrenaline, cortisol and IL-6 (Bishop and Clarke, 1998; Mitchell *et al.*, 1998; Bishop *et al.*, 2001), which have been shown to be stimulators of osteoclastogenesis and bone resorption (Kotake *et al.*, 1996; Kwan *et al.*, 2004). In the present study, PRO intakes exceeded the guideline intake for endurance athletes, whereas CHO intakes were generally lower than guideline intakes for endurance

athletes. Guideline intakes are 7 – 12 g·kgBM⁻¹·d⁻¹ for CHO and 1.6 g·kgBM⁻¹·d⁻¹ for PRO (Burke *et al.*, 2001; Tarnopolsky *et al.*, 2004; Burke *et al.*, 2011); the average intakes reported in the present study are 6 g·kgBM⁻¹·d⁻¹ for CHO and 1.8 g·kgBM⁻¹·d⁻¹ for PRO. In a study of 25 elite Australian triathletes, the average energy intake was 4,095 kcal·d⁻¹, 609 g·d⁻¹ of CHO and 133 g·d⁻¹ of PRO (Burke and Read, 1987). Taking an average body mass of 69 kg for a male triathlete, this equates to 8.8 g·kgBM⁻¹·d⁻¹ of CHO and 1.9 g·kgBM⁻¹·d⁻¹ of PRO, both of which are higher than intakes in the elite British triathletes that participated in the present study.

Micronutrient intakes are also very important for bone health, particularly calcium and vitamin D, which are related to stress fracture injury (Lappe *et al.*, 2008; Medelli *et al.*, 2009a; Nieves *et al.*, 2010; Tenforde *et al.*, 2010; Sonnevile *et al.*, 2012; Wentz *et al.*, 2012; Miller *et al.*, 2016). There is no relationship between supplementation of calcium and vitamin D and bone turnover in the present study, although the two participants with the highest PTH concentrations did not supplement with calcium. In the present study, calcium intakes averaged 1,359 ± 447 mg·d⁻¹, which is higher than the recommended intake for healthy adults (700 mg) (British Dietetics Association and the National Health Service). However, due to the large dermal calcium losses experienced by the participants during training sessions, this calcium intake is likely to be insufficient to maintain a positive calcium balance. A non-active individual will lose 15 – 60 mg in the sweat each day (National Research Council, Food and Nutrition Board; Allen, 1982; Charles *et al.*, 1983) and often dermal calcium losses are not included when calculating calcium balance and recommended intakes (Nordin and Heaney, 1990).

Taking the average calcium intake of 1,359 mg·d⁻¹ in the present study, given that approximately 30% will be absorbed in the body (Brine and Johnston, 1955; Nordin *et al.*, 1979), leaves 408 mg available, approximately 280 mg of that is excreted in faeces and urine (Heaney and Skillman, 1964; Nordin *et al.*, 1979), leaving 128 mg. The average total dermal calcium loss during a training session was 77 mg and given that these triathletes will both run and cycle at least once each day (as well as swimming) (77 * 2 = 154 mg), leaves a calcium balance of -26 mg. A negative calcium balance can cause increased bone resorption as bone is mobilised to maintain adequate serum calcium

concentrations (Brown, 1983; Brown, 2000), which may cause amplified BMC losses and contribute to low BMDs in athletes (Klesges *et al.*, 1996). Therefore, this suggests that athletes that train multiple times each day should have calcium intakes exceeding 1,400 mg·d⁻¹.

Calcium concentrations in the sweat do not vary largely between participants as sodium does (Shirreffs and Maughan, 1997). The concentration remains similar between participants (0.54 ± 0.09 mmol·L⁻¹), but the volume of sweat lost between participants varies more largely (0.79 ± 0.38 L). Therefore, an individual with higher sweat rates will lose more dermal calcium than an individual with lower sweat rates and individuals with higher sweat rates may require larger calcium intakes to replace the dermal losses. As total sweat losses and therefore total calcium losses varied depending on the type of training session performed, for example 108 ± 35 mg was lost during a long easy cycle (EB) compared to 51 ± 34 mg lost during a short but hard run (HR), calcium requirements may also vary depending on the type of training that is performed on each day. Therefore, when athletes train in environments that encourage higher sweat rates and when training volumes or intensities change, dermal calcium losses will also change. This warrants further investigation of calcium losses during different training schedules.

The rate of calcium loss also varied depending on the type of session performed; running induced a higher rate of calcium loss compared to cycling, and the more intense running sessions induced the greatest rate of calcium loss. The rate of loss may be more important than the total amount of calcium lost because of the rate dependence of the PTH response to decreased Ca²⁺ concentrations. When Ca²⁺ decreases rapidly, there is a more vigorous secretory response than when it decreases slowly (Brown, 2000). Therefore, the timing of calcium ingestion around training may be more important than the overall calcium intake across the day. However, whether the rate of dermal calcium loss causes Ca²⁺ concentrations to decrease at a similar rate requires exploration.

A negative calcium balance may contribute to the lower than average (RA) concentrations of Aca across all samples. However, serum concentrations of calcium should be tightly regulated via the actions of PTH; when Ca²⁺ decreases from the homeostatic set point, PTH is synthesised and secreted,

increasing serum calcium back to normal levels (Stuart and Broadus, 1987; Brown, 2000). This suggests that the homeostatic set point of serum calcium is lower in elite triathletes compared to recreationally active individuals. An explanation could be that maintaining a negative calcium balance over a prolonged period could reduce the homeostatic set point of serum calcium, so deviations are less likely to occur and PTH is therefore less likely to increase. This is similar to a consistent negative energy balance that suppresses metabolic processes so the body's physiological systems require less energy (Stubbs *et al.*, 2004; Loucks, 2007).

PTH is responsive to exercise and has been shown to increase by up to 84% after an acute bout of exercise, although, PTH has not been previously shown to remain elevated the morning after exercise (Scott *et al.*, 2011). In comparison, participants from site 1 showed an increase in PTH concentrations in sample 3, which was the morning after a particularly long and hard training day involving 6 – 7 hours of training. This coincided with increased PO_4 concentrations in 2 participants and a decreased ACa concentration in 1 participant. As we did not take blood samples throughout the training day we do not know whether PTH concentrations were increased after training sessions, and if so, for how long. Morning samples alone, without a baseline concentration for comparison, make it difficult to interpret the lower than average (RA) PTH concentrations in 9 out of 16 participants. The large dermal calcium losses that were likely to have occurred during this long training day could have contributed to the increase in PTH concentrations the next day (Barry *et al.*, 2011). Despite this, there was no coinciding increase in β -CTX concentrations in sample 3, suggesting that this increased PTH was not sufficient to increase bone resorption the following morning. It is possible that the lower than average resting concentrations of PTH would mean that the difference between PTH concentrations when at rest and when they are increased during exercise is greater, and this greater relative increase in PTH is likely to have the maximum anabolic effect on bone (Dempster *et al.*, 1993; Brahm *et al.*, 1997a; Brahm *et al.*, 1997b; Vainionpää *et al.*, 2009). This could contribute to the positive bone turnover marker ratios in 15 out of the 16 triathletes. However, there are no studies that have examined relative exercise-induced increases in PTH and the subsequent effect on bone turnover and structural bone adaptation, so the increase with exercise required to cause an anabolic effect is unknown. The required increase may be large, considering that therapeutic doses of PTH

are required to significantly increase bone formation in rats (Turner *et al.*, 2007) and improve BMD and fracture risk in osteoporotic men and women (Finkelstein *et al.*, 1993; Finkelstein *et al.*, 1998; Kurland *et al.*, 2000; Neer *et al.*, 2001; Orwoll *et al.*, 2003).

As well as calcium, PTH also responds to PO₄ and vitamin D. 1,25(OH)₂D₃ is the second major Ca²⁺ elevating hormone (Brown, 2013) and it mediates renal and intestinal absorption of calcium. The range of total 25(OH)D concentrations in the present study was 73 to 165 nmol·L⁻¹; therefore there were no deficiencies present and this is unlikely to have had a negative effect on calcium metabolism throughout the study period. Although, it should be stressed that there are currently no widely accepted guidelines for optimum vitamin D concentrations, especially in athletic populations (Close *et al.*, 2013). Across all samples, PO₄ concentrations were higher than average (RA) concentrations in 7 out of 16 participants and there are large variations for participants at site 1. Elevation of PO₄ concentrations, via dietary PO₄, has been shown to rapidly stimulate PTH secretion, independent of calcium or 1,25(OH)₂D₃ concentrations (Lopez-Hilker *et al.*, 1990; Estepa *et al.*, 1999; Ritter *et al.*, 2002; Martin *et al.*, 2005) and elevated PTH concentrations act to increase PO₄ resorption from the bone and decrease reabsorption in the proximal tubule (Penido and Alon, 2012). For 4 participants at site 1, higher PO₄ concentrations coincided with higher PTH concentrations in sample 3, suggesting that increased bone resorption and demineralisation could have led to higher plasma PO₄ concentrations (Berndt *et al.*, 2005; Penido and Alon, 2012). Alternatively, the higher plasma PO₄ concentrations could have stimulated the higher PTH concentrations. This highlights the need for more conclusive research concerning PTH, calcium and PO₄ regulation surrounding exercise.

The incidence of stress fracture injury, assessed by a retrospective questionnaire, was high in this group of triathletes. Twelve out of the 16 participants had suffered at least 1 stress fracture injury and 3 of these 12 participants had suffered 2 stress fractures. This is higher than the 4 to 37% of runners having suffered from a stress fracture that is reported in other studies (Hulkko and Orava 1987; Barrow and Saha, 1988; Johnson *et al.*, 1994; Bennell *et al.*, 1995; Bennell *et al.*, 1996a; Bennell *et al.*, 1996b; Bennell *et al.*, 1998; Jones *et al.*, 2002; Kelsey *et al.*, 2007; Iwamoto *et al.*, 2011; Duckham *et al.*, 2012; Tenforde *et al.*, 2013; Yagi *et al.*, 2013). These studies all evaluated

stress fracture injury using different methods. Some of these studies prospectively evaluated athletes over a short time period, such as 12 months (Zernicke *et al.*, 1993; Johnson *et al.*, 1994; Bennell *et al.*, 1996b), and this method has a tendency to show lower incidences than studies that retrospectively report stress fracture injuries, such as the present study that reported incidences throughout participants' athletic careers. It is therefore difficult to compare results of different studies in detail.

In conclusion, elite triathletes have accelerated bone turnover during this off-season phase of training, characterised by resting β -CTX and PINP concentrations that are higher than average concentrations in recreationally active individuals and non-active individuals. Although cause and effect cannot be established from the results of this study, high energy expenditures, insufficient energy intakes and high rates of dermal calcium loss, may contribute to this accelerated bone turnover. However, further research is needed to investigate whether this accelerated bone turnover is always present or if it is characteristic of the training phase and is related to training volume/intensity, dietary intakes and environmental conditions. Similarly, as dermal calcium losses in this group of triathletes are much higher than losses in the normal population, further research around calcium metabolism is warranted. It should be noted that the main limitation of the study is the lack of a baseline sample, which was impossible to obtain due to participants' training schedules not incorporating any rest periods and the lack of even a single rest day. However, consideration should be given to the fact that the participants in this study compete at World Triathlon Series level or above and represent 50% of the British Triathlon World Class Performance squad. Therefore, this study sample truly represents the elite triathlete population, the results are ecologically valid and can be directly used by triathletes and coaches.

CHAPTER 5: INVESTIGATING BONE
METABOLISM IN A SUBSET OF ELITE
TRIATHLETES DURING PRE-
COMPETITION TRAINING

5.1 Introduction

The study presented in Chapter 4 showed that elite triathletes have accelerated bone turnover during off-season training compared to recreationally active and non-active individuals. This may be caused by high training loads, high energy expenditures and high dermal calcium losses, alongside low energy intakes. All of which may contribute to the high incidence of stress fracture injuries in this group of elite triathletes. These factors will differ throughout a triathlon season, due to racing schedules and environmental conditions, for example in the 2016 season there were 9 World Series races over 7 months as well as the Olympic final, where temperatures ranged from 4°C to 30°C. Therefore, the accelerated bone turnover that was shown in the study reported in Chapter 4 may not always be present to the same extent but may be related to the training load performed during off-season training or other external factors.

Training load changes throughout the triathlon season; off-season training often involves high volume, low intensity work as the athletes return to training after a short break and work on increasing overall fitness levels and endurance. As the season progresses towards competition preparation, the volume of training starts to decrease but the intensity of training increases (anecdotal information from British Triathlon coaches). Ideally, energy intakes should also change to match the demands of the changing training schedule, but in reality this is often difficult for athletes to attain (Stubbs *et al.*, 2004; Drenowatz *et al.*, 2012), meaning that energy availability may vary throughout a triathlon season.

Environmental conditions, such as levels of sunlight and the quantity and quality of solar radiation, also change throughout the season, which influences cutaneous previtamin D₃ synthesis and therefore total 25(OH)D concentrations (Webb *et al.*, 1988). Subsequently, vitamin D concentrations have been shown to change throughout an athletic season (Close *et al.*, 2013; Wolman *et al.*, 2013; Owens *et al.*, 2015; Miller *et al.*, 2016). Additionally, differing ambient temperatures and humidity levels will alter sweat rates. As the study in Chapter 4 showed that dermal calcium losses are related to sweat losses, dermal calcium losses will increase as sweat losses increase. This may mean that

calcium intake requirements and timings will also change throughout a season and will vary depending on the type of training sessions performed.

The aim of this study was to investigate whether there are changes in resting bone turnover during pre-competition training compared to off-season training in elite triathletes. Nutritional practices and dermal calcium losses were also investigated during pre-competition training and compared to off-season training to explore whether changes in these variable factors may contribute to changes in bone turnover at different phases of the season.

5.2 Methodology

5.2.1 Participants

Participants 07 – 14 (Loughborough – site 2) from Chapter 4 also participated in a second testing period (Table 12). This study was approved by the Ethical Advisory Committee at Nottingham Trent University (Application numbers 398, 409 and 413)..

Table 12. Participant characteristics. Data are mean \pm 1SD.

	Males (n = 6)	Females (n = 2)
Age (y)	24 \pm 5	23 \pm 4
Height (m)	1.80 \pm 0.07	1.66 \pm 0.02
Body mass (kg)	70.2 \pm 3.4	59.4 \pm 0.6

5.2.2 Experimental design

In this longitudinal study, participants performed their usual training load and maintained normal behaviours for the entirety of the testing period. Prior to testing, participants provided written informed consent and completed health, injury and menstrual status (females only) questionnaires (Appendix E). The testing period lasted 7 – 10 days and was completed during pre-competition training in May 2016. Fasted morning blood samples were taken between 06:30 and 07:00 prior to morning training sessions along with the collection of the first void for the measurement of urine osmolality. Blood samples were taken at precisely the same time for each participant on each occasion and were also taken at the same time to samples in the previous study reported in Chapter 4. Dermal calcium losses were measured in cycling and running training sessions. Participants completed daily diet and training logs and coaches recorded training sessions using Training Peaks (<https://www.trainingpeaks.com/>) (Appendix F). The results of the present study were compared to the results of Chapter 4.

5.2.3 Testing procedures

The study design and testing procedures were identical to those described in Section 4.2, with the exception of DLW and the stress fracture history questionnaire. The following measures were performed: morning blood and urine collection (Section 4.2.3.1), sweat collection (Section 4.2.3.2) and diet logs (Section 4.2.3.5).

5.2.4 Bone turnover markers and metabolites

Due to there being no reference ranges for resting bone turnover markers and related metabolites available for elite athletes, the results in the present study were compared to resting concentrations for recreationally active individuals (RA) that have been collected by the Musculoskeletal Physiology Research group at Nottingham Trent University (Appendix A). Average concentrations, +1 SD and -1 SD were plotted on each graph for comparison and are referred to as 'RA' concentrations throughout Sections 5.3 and 5.4. To give a numerical value to the difference between bone formation and bone resorption, the bone turnover marker ratio was calculated using the following equation: $[P1NP]/([\beta\text{-CTX}]*100)$, with a value above 1 indicating that bone turnover favours bone formation and a value below 1 indicating that bone turnover favours bone resorption. This equation was used in Lombardi *et al.* (2012) but for different bone turnover markers.

5.2.5 Statistical analysis

Once parametric assumptions were confirmed (Section 3.8), paired sample t-tests were performed to compare the results of the pre-competition testing period with those of the off-season testing period for participants 07 – 14. Cohen's *d* effect sizes were subsequently calculated for significant differences (Section 3.8).

5.3 Results

5.3.1 Bone turnover markers

5.3.1.1 β -CTX

β -CTX concentrations were higher than average (RA) concentrations in 5 out of 8 participants in all samples and did not increase or decrease in any sample. The minimum concentration was 0.34 ng mL⁻¹ and the maximum concentration was 1.55 ng mL⁻¹. Participant 13 had average β -CTX concentrations that were more than 2.5 times the average (RA) concentration (Figure 10A). Average concentrations were significantly higher during off-season training compared to pre-competition training ($P=0.020$, $d=0.40$) (Figure 12A).

5.3.1.2 P1NP

P1NP concentrations were higher than average (RA) concentrations in 4 out of 8 participants in all samples and did not increase or decrease in any sample. The minimum concentration was 42.8 ng mL⁻¹ and the maximum concentration was 158.8 ng mL⁻¹. Participant 13 had average P1NP concentrations that were 2.5 times the average (RA) concentration (Figure 10B). Average concentrations were significantly higher during off-season training compared to pre-competition training ($P=0.012$, $d=0.40$) (Figure 12B).

5.3.1.3 Bone turnover marker ratios

Although 5 out of the 8 participants showed an increase in the bone turnover marker ratio from off-season to pre-competition training, the increase was not significant (Table 13).

Table 13. Average bone turnover marker ratios.

Participant	Bone turnover marker ratio Off-season	Bone turnover marker ratio Pre-competition	Percentage change in the bone turnover marker ratio
07	1.23	1.29	4.87
08	1.44	1.03	-28.47
09	0.82	0.92	12.19
10	1.11	1.11	0.00
11	1.04	0.93	-10.57
12	1.07	1.12	4.67
13	1.04	1.19	14.42
14	1.17	1.20	2.56

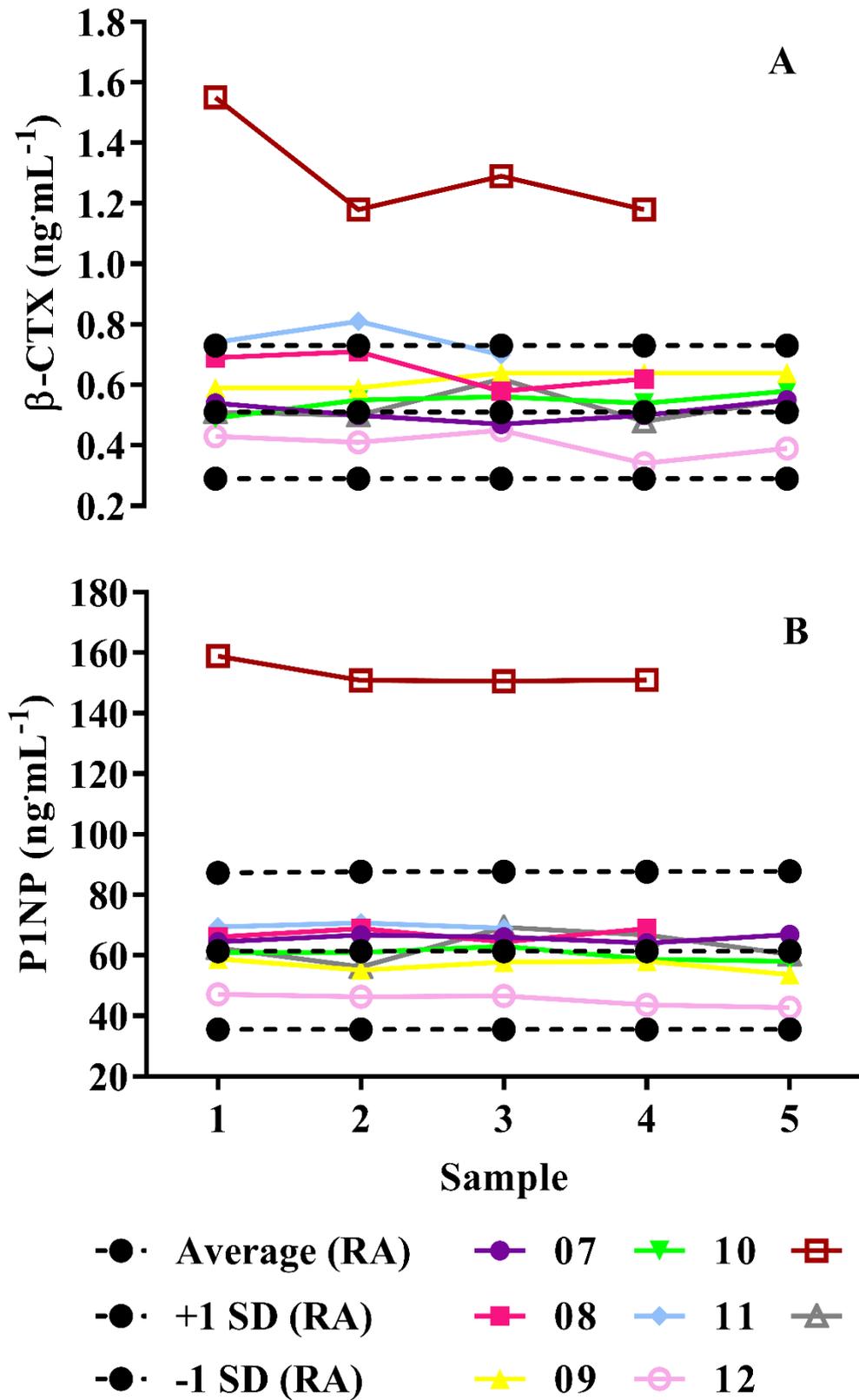


Figure 10. Resting concentrations of β -CTX (A) and PINP (B) for all participants in all blood samples over 7 – 10 days during pre-competition training. See Figure 12 for comparisons between off-season training and pre-competition training.

5.3.2 Calcium metabolism

5.3.2.1 PTH

PTH concentrations were lower than average (RA) concentrations in 5 out of 8 participants and higher than average (RA) concentrations in 3 out of 8 participants in all samples. Concentrations increased in sample 2 in 5 out of 8 participants, which was the morning after an evening track running session. Concentrations also increased in sample 5 in 2 out of 8 participants. The minimum concentration was $2.10 \text{ pmol}\cdot\text{L}^{-1}$ and the maximum concentration was $4.80 \text{ pmol}\cdot\text{L}^{-1}$. The PTH concentration in sample 2 was almost 1.5 times the average (RA) concentration in participant 07 (Figure 11A). Average concentrations were not significantly different between off-season training and pre-competition training ($P=0.404$) (Figure 12C).

5.3.2.2 ACa

ACa concentrations were lower than average (RA) concentrations in 8 out of 8 participants in all samples. The minimum concentration was $2.18 \text{ mmol}\cdot\text{L}^{-1}$ and the maximum concentration was $2.37 \text{ mmol}\cdot\text{L}^{-1}$ (Figure 11B). Average concentrations were significantly higher during off-season training compared to pre-competition training ($P\leq 0.001$, $d=2.60$) (Figure 12D).

5.3.2.3 PO₄

PO₄ concentrations were lower than average (RA) concentrations in 5 out of 8 participants in all samples. Concentrations decreased in sample 2 and increased in sample 4 in participant 09 and increased in sample 4 in participant 14. The minimum concentration was $0.87 \text{ mmol}\cdot\text{L}^{-1}$ and the maximum concentration was $1.35 \text{ mmol}\cdot\text{L}^{-1}$ (Figure 11C). Average concentrations were significantly higher during off-season training compared to pre-competition training ($P=0.050$, $d=0.82$) (Figure 12E).

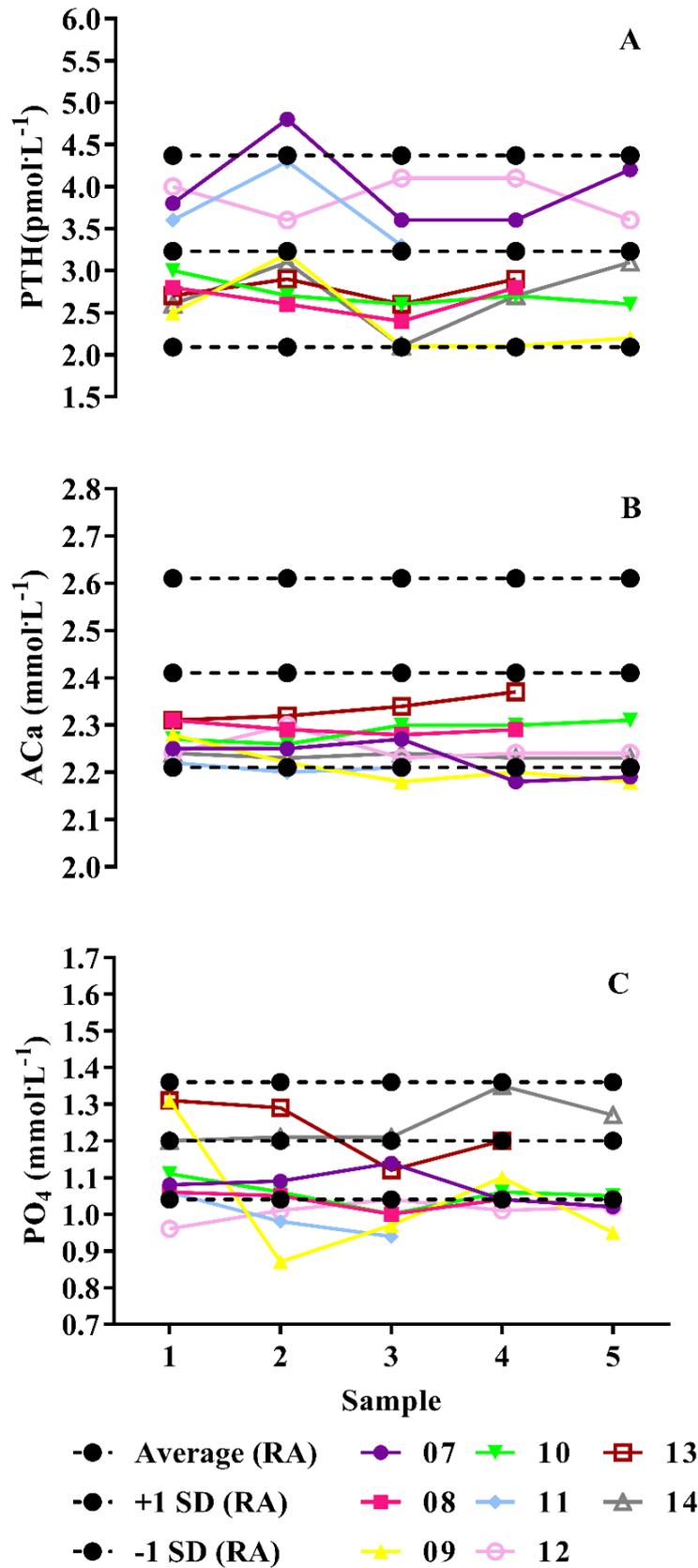


Figure 11. Resting concentrations of PTH (A), ACa (B) and PO₄ (C) for all participants in all blood samples over 7 – 10 days during pre-competition training. See Figure 12 for comparisons between off-season training and pre-competition training.

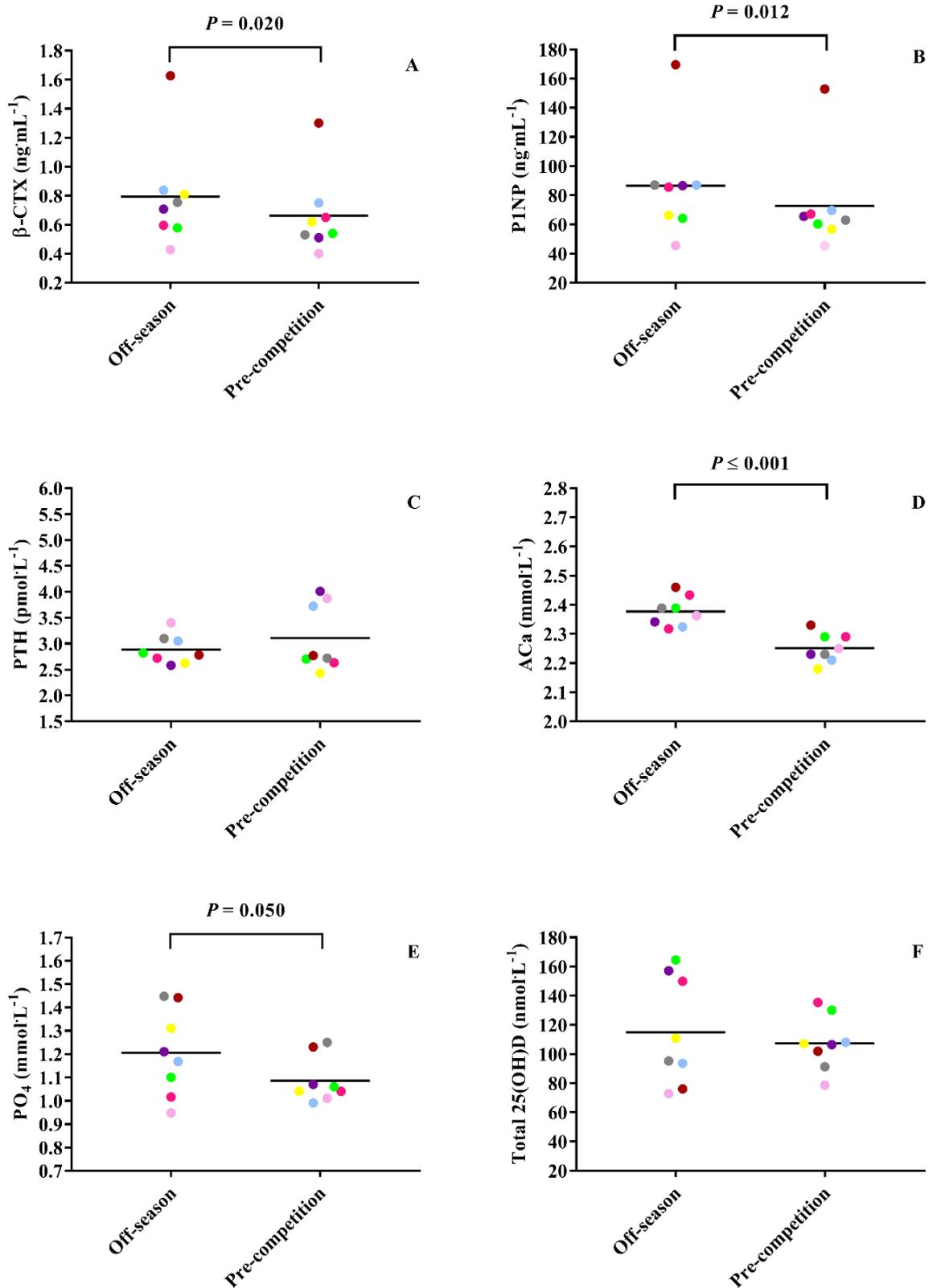


Figure 12. Average concentrations of β -CTX (A), P1NP (B), PTH (C), A Ca (D), PO₄ (E) and total 25(OH)D (F) during off-season and pre-competition training. Each coloured circle represents average concentrations over all samples for each participant and the horizontal bars represent the average concentrations for all participants. Significant differences are presented on each graph.

5.3.3 Vitamin D

All participants had sufficient total 25(OH)D concentrations, despite cessation of supplementation. Deficient concentrations are 50 nmol·L⁻¹ or less, low optimal concentrations are 75 nmol·L⁻¹ and high optimal concentrations are 125 nmol·L⁻¹, according to the English Institute of Sport's and British Triathlon's recommended guidelines for athletes. The minimum concentration was 73.6 nmol·L⁻¹ and the maximum concentration was 140.3 nmol·L⁻¹ (Figure 13). Average concentrations were not significantly different between off-season and pre-competition training ($P=0.417$) (Figure 12F).

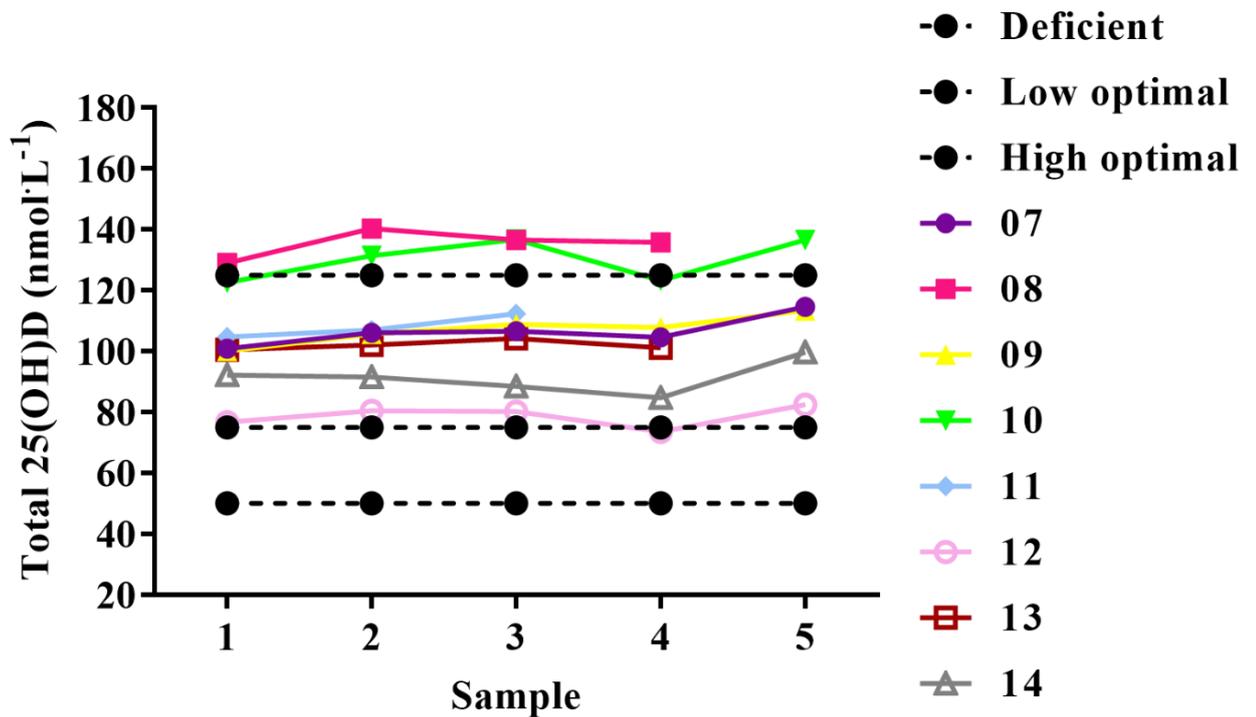


Figure 13. Resting concentrations of total 25(OH)D for all participants in all blood samples over 7 – 10 days during pre-competition training. See Figure 12 for comparisons between off-season training and pre-competition training.

5.3.4 Energy intakes

Average daily energy (kcal), CHO (g), PRO (g) and calcium (mg) intakes were significantly higher during pre-competition training compared to off-season training ($P \leq 0.005$, $d = 2.70$ to 3.80) (Table 14) (Figure 14A – D). Average daily CHO intakes, expressed as a percentage of total intake, were significantly higher during pre-competition training compared to off-season training ($P \leq 0.05$, $d = 1.50$), PRO intakes, expressed as a percentage of total intake, were not significantly different between training phases ($P = 0.44$, $d = 1.00$).

Table 14. Average daily energy, CHO, PRO and calcium intakes and urine osmolality for all participants during pre-competition training. Data are averaged from a minimum of 5 day diet logs and 5 urine samples. Diet logs are missing for participants 09 and 13. Participants were classed as hydrated at ≤ 500 mOsmL⁻¹, dehydrated at 501 – 999 mOsmL⁻¹ and severely dehydrated at ≥ 1000 mOsmL⁻¹.

Participant	Sex	Body mass (kg)	Average	Average	Average	Average	Average	Average	Average
			daily energy intake (kcal)	daily carbohydrate intake (g)	daily carbohydrate intake (% of total intake)	daily protein intake (g)	daily protein intake (% of total intake)	daily calcium intake (mg)	daily urine osmolality (mOsmL ⁻¹)
07	F	59.9	2,824	423	60	150	21	1,850	670
08	M	74.1	3,926	543	55	193	20	2,077	735
09	M	71.3	N/A	N/A	N/A	N/A	N/A	N/A	1075
10	M	70.8	3,756	467	50	175	19	2,171	1018
11	M	65.2	3,823	529	55	190	20	1,406	743
12	F	59.0	4,118	591	57	169	16	2,587	520
13	M	73.0	N/A	N/A	N/A	N/A	N/A	N/A	990
14	M	67.1	3,808	501	53	148	16	2,130	956
Mean ± SD		67.6 ± 5.8	3,709 ± 452	509 ± 59	55 ± 4	171 ± 19	19 ± 2	2,037 ± 391	838 ± 198

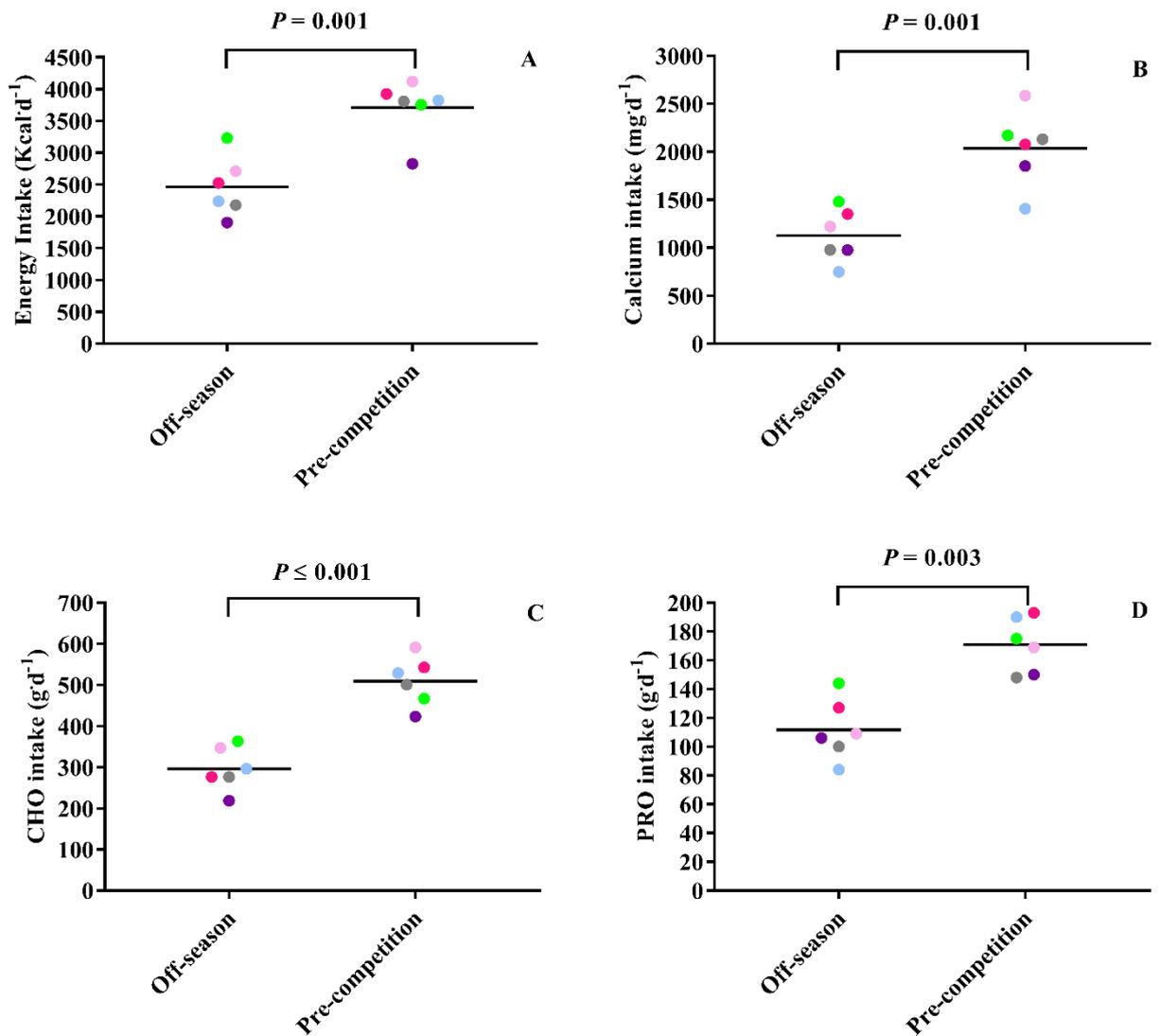


Figure 14. Average daily intakes of total energy (A), calcium (B), CHO (C), and PRO (D) during off-season and pre-competition training. Each coloured circle represents average intakes from a minimum of 5 day diet logs for each participant and the horizontal bars represent the average intakes for all participants. Significant differences are presented on each graph. Diet logs are missing for participants 09 and 13 in pre-competition training and have therefore been excluded from the statistical analysis.

5.3.5 Supplementation

Other than participant 07, who was taking 534 mg of calcium 3 times per week, no other participants were taking any supplements.

5.3.6 Training loads

Average time spent swimming, cycling, running and strength and conditioning was not significantly different between off-season and pre-competition training ($P=0.058$ to 0.461). Participants 08, 11 and 13 had a forthcoming World Series race, so had started to taper towards the end of the week (Table 15).

Table 15. Total training time, minutes of swimming, cycling, running and strength and conditioning (S&C) for all participants during 7 days of pre-competition training.

Participant	Total swim time over 7 days (mins)	Total cycling time over 7 days (mins)	Total run time over 7 days (mins)	S&C over 7 days (mins)	Total over 7 days (mins)
07	405	810	360	225	1,800
08	315	720	300	225	1,560
09	405	810	360	225	1,800
10	405	810	360	225	1,800
11	315	720	300	225	1,560
12	405	810	360	225	1,800
13	315	720	300	225	1,560
14	405	810	360	225	1,800
Mean ± SD	317 ± 47	776 ± 47	338 ± 31	225 ± 0	1,710 ± 124

5.3.7 Sweat losses and dermal calcium losses

Table 16. Total sweat and calcium losses and rates of sweat and calcium losses for all participants.

The average concentration of calcium in sweat was $0.56 \pm 0.06 \text{ mmol}\cdot\text{L}^{-1}$. Data are mean \pm 1SD.

	Training session			
	ER	EB	HR	HB
Total sweat loss (L)	1.0 ± 0.3	1.4 ± 0.7	1.0 ± 0.5	1.1 ± 0.3
Total calcium loss (mg)	90 ± 33	147 ± 60	109 ± 59	108 ± 42
Rate of sweat loss (L·h ⁻¹)	0.79 ± 0.22	0.49 ± 0.15	1.47 ± 0.51	0.80 ± 0.18
Rate of calcium loss (mg·h ⁻¹)	75 ± 24	47 ± 16	155 ± 59	82 ± 26

Average total calcium loss, total sweat loss, rate of calcium loss and rate of sweat loss were significantly higher during pre-competition training compared to off-season training ($P \leq 0.01$, $d = 1.25$ to 1.60) (Figure 15A – D).

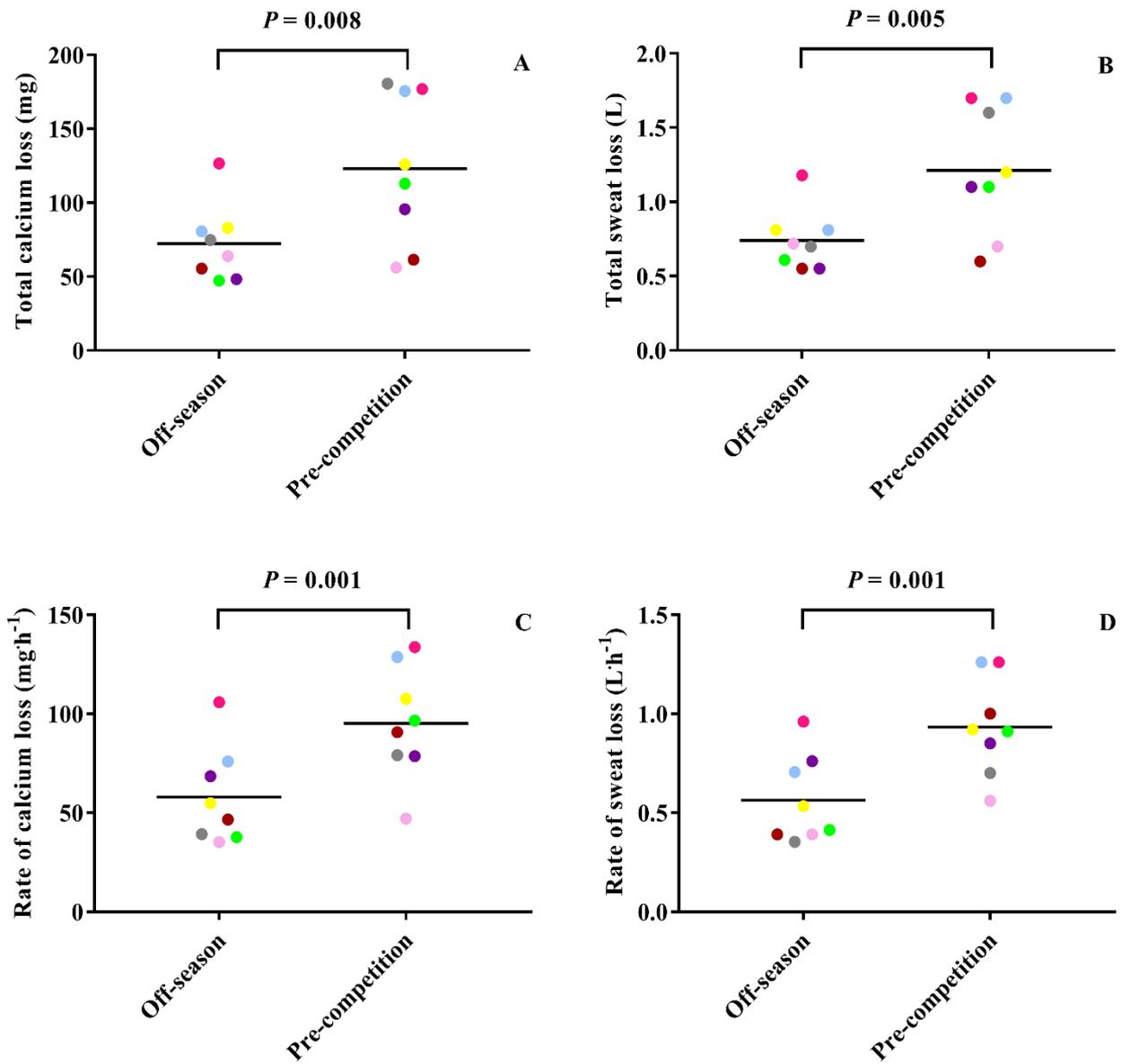


Figure 15. Average total calcium loss (A), average total sweat loss (B), average rate of calcium loss (C) and average rate of sweat loss (D) during off-season and pre-competition training. Each coloured circle represents average total losses or rate for each participant and the horizontal bars represent the average losses or rate for all participants. Significant differences are presented on each graph.

5.2.8 Menstrual status

The two female participants were using combined oral contraception (both Levest).

5.4 Discussion

The main findings of the study are: 1) β -CTX concentrations were consistently higher than average (RA) concentrations in 5 out of 8 participants and average β -CTX concentrations were higher during off-season training compared to pre-competition training; 2) P1NP concentrations were consistently higher than average (RA) concentrations in 4 out of 8 participants and average P1NP concentrations were higher during off-season training compared to pre-competition training; 3) PTH concentrations were lower than average (RA) concentrations in 5 out of 8 participants and higher than average in 3 out of 8 participants; 4) Aca concentrations were consistently lower than average (RA) concentrations in 8 out of 8 participants and average Aca concentrations were higher during off-season training compared to pre-competition training; 5) PO_4 concentrations were lower than average (RA) concentrations in 5 out of 8 participants and average PO_4 concentrations were higher during off-season training compared to pre-competition training; 6) Average daily energy, calcium, CHO and PRO intakes were higher during pre-competition training compared to off-season training; 7) Average total calcium loss, total sweat loss, rate of calcium loss and rate of sweat loss were higher during pre-competition training compared to off-season training.

To our knowledge this is the first study that has investigated bone turnover in elite triathletes at two phases of the season. Maïmoun *et al.* (2004b) studied 7 male sub-elite triathletes, at the start of the season and 32 weeks later, taking measures of BALP, OC and u-CTX. There was a decrease in BALP concentrations, but no other differences. Two isolated measurements are unlikely to capture the dynamic nature of bone turnover throughout a triathlon season, whereas, in the present study, we made 5 measurements in each training phase. In the present study, the average concentrations of β -CTX ($0.64 \pm 0.26 \text{ ng}\cdot\text{mL}^{-1}$) and P1NP ($70.64 \pm 30.44 \text{ ng}\cdot\text{mL}^{-1}$) were high compared to non-active (β -CTX; $0.23 - 0.32 \text{ ng}\cdot\text{mL}^{-1}$ and P1NP; $31.00 - 49.40 \text{ ng}\cdot\text{mL}^{-1}$) and recreationally active individuals (β -CTX; $0.51 \pm 0.22 \text{ ng}\cdot\text{mL}^{-1}$ and P1NP; $61.40 \pm 25.89 \text{ ng}\cdot\text{mL}^{-1}$) but were not as high as average concentrations during off season training (β -CTX; $0.80 \pm 0.36 \text{ ng}\cdot\text{mL}^{-1}$ and P1NP; $86.43 \pm 36.11 \text{ ng}\cdot\text{mL}^{-1}$ in participants 07 – 14).

Similarly to off-season training, bone turnover marker concentrations were higher than average (RA) in all samples in 4 out of 8 participants; there were no increases and decreases on certain mornings. The significantly lower concentrations of both β -CTX and P1NP during pre-competition training compared to off-season training suggests that bone turnover is not accelerated to the same extent. Lombardi *et al.* (2011) showed that bone formation markers BALP and OC and marker of osteoclast activity TRAP5b, were significantly increased from the end of the pre-competitive season to the end of the competitive season in 14 female elite slalom skiers. This was attributed to the competitive season being more physically demanding because of higher loads of weight bearing activity. Likewise, the results of the study reported in Chapter 4 and the present study also showed that bone turnover is higher after high volumes of training. Although we were unable to adequately measure training intensity due to a lack of detail provided by coaches and athletes, anecdotal information from British Triathlon coaches and support staff shows that the number of high volume, low intensity sessions decreases and the number of low volume, high intensity sessions increases as triathletes move from off-season to pre-competition training. Shorter bouts of higher intensity training are anabolic for bone, compared to lower intensity, prolonged training (O'Connor *et al.*, 1982; Rubbin and Lanyon, 1984; Rubin and Lanyon, 1985; Raab-Cullen *et al.*, 1994).

Similarly to the off-season testing period, participant 13 had β -CTX and P1NP concentrations that were 2.5 times higher than the average (RA) concentrations, with peak β -CTX concentrations of 1.55 ng·mL⁻¹ and peak P1NP concentrations of 158.80 ng·mL⁻¹. However participant 13 showed an increase in the bone turnover marker ratio from 1.04 to 1.19. Likewise, in 5 of the 8 participants, the bone turnover marker ratio increased from off-season training to pre-competition, suggesting that bone turnover is favouring bone formation more during pre-competition training.

Different sports induce different patterns of mechanical loading and different physiological responses (Maimoun *et al.*, 2004b; Hermann *et al.*, 2007; Mouzopoulos *et al.*, 2007; Lippi *et al.*, 2008; Lombardi *et al.*, 2011), some of which will be more osteogenic than others. Further, study protocols vary widely even in the limited studies that have investigated the variations of bone turnover markers in elite athletes during a season. This means that the results of different studies

cannot be directly compared and highlights the need for a standard study protocol to be developed to allow for better comparison between different athletes and sports.

Total energy, CHO (g) and PRO (g) intakes were significantly higher during pre-competition training compared to off-season training. Intakes of CHO as a percentage of total intake, were significantly higher in pre-competition training compared to off-season training ($55 \pm 4\%$ vs $49 \pm 4\%$), however, intakes of PRO as a percentage of total intake, were not different between training phases ($19 \pm 2\%$ vs $17 \pm 2\%$). The relative increase in CHO and relative decrease in fat intake during the pre-competition training phase, could have contributed to a more anabolic environment for bone, as a low CHO intake can increase osteoclast activity and bone resorption and increase stress hormone and cytokine responses to exercise (Hou *et al.*, 1990; Li *et al.*, 1990; Zernicke *et al.*, 1995; Bishop and Clarke, 1998; Mitchell *et al.*, 1998; Wohl *et al.*, 1998; Bishop *et al.*, 2001; Kettler, 2001; Bielohuby *et al.*, 2010). The increased CHO intakes could have also meant that the triathletes were more optimally fuelled to perform the high intensity training sessions, as CHO (muscle glycogen and plasma glucose) is the main fuel source for high intensity exercise (van Loon *et al.*, 2001).

The difference in average energy intakes between off-season and pre-competition training was $1,334 \pm 312 \text{ kcal}\cdot\text{d}^{-1}$ for the male triathletes and $1,164 \pm 346 \text{ kcal}\cdot\text{d}^{-1}$ for the female triathletes. This makes it more likely that energy intakes were more closely matched to energy expenditures, providing more energy to adequately support training and improving training fuelling and recovery. Despite this, if energy expenditures were still in the region of $5,500 - 6,000 \text{ kcal}\cdot\text{d}^{-1}$ for males and $4,000 - 5,000 \text{ kcal}\cdot\text{d}^{-1}$ for females, the triathletes may still be in negative energy balance during pre-competition training. Even a small energy deficit has been shown to affect bone turnover; Ihle and Loucks (2004) have shown that bone formation is suppressed when energy availability is reduced to only $30 \text{ kcal}\cdot\text{kgLBM}^{-1}\cdot\text{d}^{-1}$, which is common even in regularly menstruating female athletes (Thong *et al.*, 2000). Because of the implications of a negative energy balance, but the apparent inability of triathletes to consume sufficient food throughout the day, alternative nutritional practices that favourably affect bone turnover need to be investigated. It should be noted that participants were

briefed on the issues with under-reporting of diet logs prior to participating in the present study, making under-reporting less likely in this second testing period.

The present study adds to the evidence of RED-S occurring in male athletes, as the results show that elite male endurance athletes have high bone turnover and low energy intakes at two phases of the season. Studies at other phases of the season, such as during and after the competition period are now required to determine whether this high bone turnover and low energy intakes are maintained all year round, and should also be linked to changes in indices of bone health, such as BMD or bone structure. One of the female participants (participant 07) is likely to have low energy availability, considering that her average energy intake was only 2,828 kcal·d⁻¹. Further, this participant had an average BMD of 1.0805 g·cm⁻² and a Z-score of 0.2, taken from two DXA scans in April and July. Considering that athletes involved in weight-bearing sports should have a 5 – 30% greater BMD than non-athletes, due to positive adaptation to mechanical loading, the BMD of participant 07 is low and should be between 1.134 and 1.404 g·cm⁻² (Fehling *et al.*, 1995; Bennell *et al.*, 1997; Nichols *et al.*, 2000; Nichols *et al.*, 2007; Tenforde *et al.*, 2015). This potentially low energy availability and low BMD suggest that the triad may be present in this female triathlete. Although both female participants were using hormonal contraception which can mask amenorrhea (Baird and Glasier, 1993; Grinspoon *et al.*, 2003), so natural oestradiol concentrations and menstrual status are unknown.

Calcium intakes significantly increased during pre-competition training, from 1,155 mg·d⁻¹ to 2,037 mg·d⁻¹, which primarily came from an increase in dairy foods such as milk and yoghurt, although participant 07 did also start to supplement with calcium. These intakes should provide an adequate supply of calcium, which should allow serum calcium levels to be maintained without the need for mobilising bone stores to raise serum calcium concentrations (McSheehy and Chambers, 1986; Thorsen *et al.*, 1997; Brown 2000; Zitterman *et al.*, 2002; DeLuca, 2004; Barry *et al.*, 2011; Brown, 2013). Resting concentrations of ACa are significantly lower during pre-competition training compared to off-season training, suggesting that the homeostatic set point of serum calcium has decreased. Average rates of dermal calcium loss and total dermal calcium losses increased significantly from off-season training to pre-competition training, which could be related to the

higher sweat rates or the higher dietary calcium intakes. There is a limit to how much calcium the body can absorb, before the rest is excreted from the body (Nordin and Heaney, 1990) and with lower intakes, calcium absorption becomes more efficient and urinary losses decrease (Weaver and Heaney, 2006). However, Martin *et al.* (2007) showed that although urinary and faecal calcium excretion increased when individuals supplemented with calcium, overall calcium retention was higher and resulted in a positive calcium balance, whereas, with low calcium intakes, retention was poor and calcium balance was negative. This suggests that the triathletes were more likely to be in a positive calcium balance during pre-competition training, but the larger total dermal calcium losses and higher rates of dermal calcium loss outweigh the higher dietary calcium intakes. It may also mean that the timing of calcium intakes around certain training sessions (e.g. hard run) may be more important for maintaining acute calcium homeostasis, compared to total dietary calcium intakes across the day.

All participants had the highest rates of calcium loss during the hard run, which may be related to the highest rates of sweat loss. This confirms the findings of Consolazio *et al.* (1962), who showed that the daily total calcium in sweat increased as the sweat rate increased in men, and the authors concluded that calcium requirements may therefore increase under profuse sweating conditions. The rates of dermal calcium loss reported in Chapter 4 and the present study are similar to rates reported in studies that collected sweat during 2 hours of indoor cycling; $69 \pm 36 \text{ mg}\cdot\text{h}^{-1}$ (Barry and Khort, 2007) and $68 \pm 30 \text{ mg}\cdot\text{h}^{-1}$ (Barry and Khort, 2008). Only the hard run induced rates similar to rates reported during an indoor 35 km cycling time trial ($120 - 147 \text{ mg}\cdot\text{h}^{-1}$) (Barry *et al.*, 2011). Considering these studies were performed indoors, the ambient temperatures were likely to be higher than in the study reported in Chapter 4 and the present study (7.1°C and 15.6°C on average – Appendix D) and they would also have no air flow, although ambient conditions are not reported in these studies. This should mean that the sweat rates and therefore the rates of dermal calcium loss would be higher during the indoor cycling compared to real-life, outdoor training sessions. It is important to explore the significance of the greater rate of loss during a hard-running session compared to other training sessions, because this could cause a much greater perturbation in calcium homeostasis and therefore a greater PTH and bone resorption response (Brown, 2000).

Despite the lower ACa concentrations, there was no significant difference in average PTH concentrations between off-season and pre-competition training, however, there was more variation in the concentrations throughout the pre-competition testing period. PTH concentrations increased in sample 2 in 5 out of 8 participants, which was the morning after an evening track running session. As we did not take blood samples throughout the training day we do not know whether PTH concentrations were increased after this track session. It was a 30–35 minutes, high intensity running session, which has previously been shown to cause transient spikes in PTH concentrations (Scott *et al.*, 2011) that are likely to be anabolic for bone (Tam *et al.*, 1982; Dempster *et al.*, 1993; Frolik *et al.*, 2003). These potential transient increases in PTH concentrations caused by the high intensity training sessions could contribute to the increased bone turnover marker ratios in pre-competition training; however, further research is needed to confirm this.

In conclusion, bone turnover is accelerated in elite triathletes during pre-competition training, characterised by resting β -CTX and P1NP concentrations that are higher than average concentrations in recreationally active individuals and non-active individuals. However, bone turnover is not as high as it is during off-season training. This is not likely to be influenced by rest, as the participants did not have any rest periods in between the testing periods. Therefore the lower bone turnover during pre-competition training may be due to increased total energy, calcium, CHO and PRO intakes in pre-competition training compared to off-season training. The rate of calcium loss is highest during hard running sessions in both phases of the season, which suggests that the timing of calcium ingestion around this type of training session may be more important than overall calcium intakes. However, there is still limited data surrounding PTH regulation during exercise, the timeframe of the response and whether this is solely mediated by changes in calcium.

CHAPTER 6: EXPLORING THE MECHANISM OF PARATHYROID REGULATION DURING EXERCISE AND RECOVERY IN RECREATIONAL RUNNERS

This study has been published in the Journal of Clinical Endocrinology and Metabolism and has been presented at the American College of Sports Medicine Annual Conference:

Townsend, R., Elliott-Sale, K. J., Pinto, A. J., Thomas, C., Scott, J. P., Currell, K., Fraser, W. D., & Sale, C. (2016). Parathyroid Hormone Secretion Is Controlled by Both Ionized Calcium and Phosphate During Exercise and Recovery in Men. *The Journal of Clinical Endocrinology & Metabolism*, *101*(8), 3231-3239. (Appendix N).

Townsend, R., Elliott-Sale, K. J., Pinto, A. J., Currell, K., Fraser, W. D., & Sale, C. (2016). Parathyroid Hormone (PTH) Secretion is Controlled by Both Ionised Calcium and Phosphate During Exercise and Recovery: 691 Board #7 June 1, 3: 30 PM-5: 00 PM. *Medicine and Science in Sports and Exercise*, *48*(5 Suppl 1), 184.

6.1 Introduction

The results of the studies reported in Chapters 4 and 5 showed that elite triathletes lose large amounts of calcium in sweat, that are higher than the normal population and similar to trained cyclists (National Research Council, Food and Nutrition Board; Consolazio *et al.*, 1962; Allen, 1982; Charles *et al.*, 1983; O'Toole *et al.*, 2000; Barry and Khort, 2007; Barry and Khort, 2008; Barry *et al.*, 2011). The rate of calcium lost in the sweat was highest during hard running sessions ($155 \pm 59 \text{ mg}\cdot\text{h}^{-1}$) and this may cause a rapid PTH response, due to the rate dependence of the PTH response to decreased Ca^{2+} concentrations (Brown, 2000). Furthermore, negative calcium balances may be prevalent at some phases of the triathlon season due to these large dermal calcium losses, which are exacerbated by insufficient calcium intakes to replace the losses (Chapter 4) (Waldron-Lynch *et al.*, 2010). A positive calcium balance is necessary for bone adaptation to mechanical loading (Lappe *et al.*, 2008).

Barry *et al.* (2011) hypothesised that the disruption of calcium homeostasis is a potential mediator of bone loss, based on the findings that sweating during exercise causes large dermal calcium losses, leading to an increase in PTH, which can subsequently cause an increase in bone resorption (Klesges *et al.*, 1996; Barry and Kohrt, 2007; Barry and Khort, 2008). Given the potential negative consequences of an imbalance in bone turnover and constant deviations from calcium homeostasis, which may be in part caused by large dermal calcium losses, it is pertinent that interventions around serum calcium maintenance during exercise are investigated in elite triathletes, similar to previous studies by Guillemant *et al.* (2004) and Barry *et al.* (2011).

Guillemant *et al.* (2004) investigated the effect of calcium ingestion in 12 trained triathletes. The authors demonstrated that when 60 minutes of cycling was performed without a calcium load before or during the exercise, β -CTX concentrations increased progressively 30 minutes after the start of the exercise and that concentrations were still significantly elevated by 45 – 50%, 2 hours after the exercise. PTH concentrations increased 2.5 – 3 fold during the exercise. In contrast, when participants consumed a total of 972 mg of calcium, in fractionated amounts every 15 minutes from 2 hours prior to exercise until the end of exercise, the increase in β -CTX concentrations was completely suppressed. The increase in PTH was only partially suppressed, therefore the regulation

of PTH during exercise and recovery could also involve other metabolites such as PO_4 (Martin *et al.*, 2005; Scott *et al.*, 2011; Scott *et al.*, 2012; Scott *et al.*, 2014). However, the mechanism and timeframe of calcium and PTH regulation during exercise and recovery is still not fully understood and needs to be explored in detail before any interventions are implemented in elite athletes.

At rest, PTH secretory activity is regulated by serum Ca^{2+} , which is detected by the calcium-sensing receptor on the chief cells of the parathyroid gland (Brown, 2000). When Ca^{2+} decreases from the homeostatic set point, PTH is synthesised and secreted, increasing serum calcium through mobilisation of the bone reservoir via bone resorption, and by increasing renal tubular reabsorption and intestinal calcium absorption (McSheehy and Chambers, 1986; Thorsen *et al.*, 1997; Zitterman *et al.*, 2002). PTH has a dual effect on bone that appears to be primarily determined by the signalling mechanism and the length of time that concentrations remain elevated above baseline and secondarily by the maximum PTH concentration (Frolik *et al.*, 2003). Prolonged elevations above baseline (approximately 2-fold), that can be induced by continuous infusion of PTH (Frolik *et al.*, 2003) or by endurance type exercise (Ljunghall *et al.*, 1986; Ljunghall *et al.*, 1988), can result in the loss of the circadian rhythm of PTH (Tsai *et al.*, 1984; Chappard *et al.*, 2001) and cause an increase in bone resorption (Tam *et al.*, 1982; Hock and Gera, 1992; Uzawa *et al.*, 1995). Whereas, transient spikes in PTH, that can be induced by intermittent administration of PTH, high intensity interval type training or acute bouts of exercise (Scott *et al.*, 2010; Scott *et al.*, 2011; Scott *et al.*, 2012; Scott *et al.*, 2013; Scott *et al.*, 2014), can cause an increase in bone formation (Tam *et al.*, 1982; Hock and Gera, 1992; Uzawa *et al.*, 1995). Chronic elevations in PTH concentrations have been associated with increased fracture risk (Sakuma *et al.*, 2006; Välimäki *et al.*, 2005), which are debilitating injuries for elite athletes (Ranson *et al.*, 2010). Therefore, understanding how PTH is regulated during exercise and recovery may have implications for endurance athletes that are at risk of chronically elevated PTH concentrations.

Exercise increases PTH concentrations (Bouassida *et al.*, 2003; Maïmoun *et al.*, 2006; Barry and Khort 2007; Herrmann *et al.*, 2007; Scott *et al.*, 2010; Barry *et al.*, 2011; Scott *et al.*, 2011; Scott *et al.*, 2012; Scott *et al.*, 2013; Scott *et al.*, 2014), although studies have used different exercise modes,

durations and intensities. Exercise intensity is important, given that Scott *et al.* (2011) has shown that 60 minutes of running at 55%, 65% and 75% of VO_{2max} results in different PTH responses during and after exercise. Therefore, any study investigating the underlying mechanisms responsible for the changes in PTH during exercise and recovery should examine the effects of exercise intensity. There may also be a different PTH response to different types of exercise, such as cycling and running, due to the different physiological responses and differences in mechanical loading. To date, this has not been investigated, but running was chosen in the present study due to the higher rate of dermal calcium loss shown during running (Chapters 4 and 5) and the higher risk of stress fracture injury in runners than cyclists (Maïmoun *et al.*, 2004b; Rector *et al.*, 2008; Scofield and Hecht, 2012).

During exercise, reductions in circulating calcium do not explain the increase in PTH, as the concentration of ACa – a surrogate for Ca^{2+} – is either increased (Maïmoun *et al.*, 2006; Scott *et al.*, 2010; Scott *et al.*, 2011) or unchanged (Barry and Khort, 2007; Scott *et al.*, 2012; Scott *et al.*, 2013) concomitantly with PTH. Barry *et al.* (2011) showed that calcium ingestion before exercise attenuated, but did not prevent the increase in PTH, suggesting that some other mechanism contributed to the increase. This could involve PO_4 , as an increase in PO_4 increases PTH in rested individuals (Martin *et al.*, 2005). Following exercise, PO_4 concentrations decrease and the timing and magnitude of these decreases reflect those in PTH (Scott *et al.*, 2011, Scott *et al.*, 2012, Scott *et al.*, 2014), also suggesting that PO_4 may be involved in PTH regulation during exercise.

The hypothesis that decreased Ca^{2+} triggers increased PTH during exercise has not yet been proven (Barry *et al.*, 2011). PTH is secreted within seconds of a decrease in Ca^{2+} and subsequent increases in Ca^{2+} take only minutes to occur in response to increased PTH, highlighting a dynamic relationship (Brown, 1983; Brown, 2000). Despite this, no studies have measured PTH and other markers of calcium metabolism until 20 minutes of exercise has been completed, by which time PTH is elevated. Most studies have started taking measurements at 30 minutes post-exercise, by which time PTH has returned to near pre-exercise levels (Guillemant *et al.*, 2004; Scott *et al.*, 2010; Barry *et al.*, 2011; Scott *et al.*, 2011; Scott *et al.*, 2012; Scott *et al.*, 2013). Single or infrequent measurements of PTH, ACa and PO_4 during and after exercise might fail to capture the dynamic nature of calcium regulation

with exercise (Barry *et al.*, 2011). The aim of this study was to use repeated measurements with a high frequency to examine the temporal pattern of PTH, PO₄, ACa and Ca²⁺ during and after 30 minutes of treadmill running at three exercise intensities.

6.2 Methodology

6.2.1 Participants

Ten healthy, recreational male runners ([mean \pm 1SD] age 23 ± 1 y, height 1.82 ± 0.07 m, body mass 77.0 ± 7.5 kg) volunteered for the study, which was approved the Ethical Advisory Committee at Nottingham Trent University (Application number 338). Participants were non-smokers, had not suffered a fracture in the past 12 months, were free from musculoskeletal injury and were not taking any medication or experiencing any problems known to affect calcium or bone metabolism. Eligibility was confirmed during the initial session, when participants provided written informed consent.

6.2.2 Experimental design

This was a randomised, counterbalanced, crossover study. Participants completed a preliminary visit for health screening, habituation and measurement of VO_{2max} . Participants then completed three randomised (Latin Square Design), three-day experimental trials, each separated by one week. On days 1 – 2, participants refrained from exercise, caffeine and alcohol. On day 2, participants consumed a self-selected diet that was repeated before each trial. On day 3, participants performed a 30 minute bout of running at 55%, 65% and 75% VO_{2max} , followed by 2.5 h of recovery.

6.2.3 Trial procedures

6.2.3.1 Assessment of VO_{2max}

Participants performed an incremental treadmill test to determine lactate threshold, followed by a ramp test to determine VO_{2max} (Section 3.2.1). The level running velocities corresponding to 55% (8.7 ± 0.6 km·h⁻¹), 65% (10.1 ± 0.8 km·h⁻¹) and 75% VO_{2max} (11.9 ± 0.9 km·h⁻¹) were calculated based on the regression of VO_2 and velocity.

6.2.4 Main trials

Participants arrived in the laboratory at 08:30 following an overnight fast from 20:00 the previous

evening and after consuming 500 mL of water upon awakening. After voiding, participants had their body mass measured before adopting a semi-recumbent position and having a cannula inserted into a forearm vein (Section 3.3.2.3). After 10 minutes rest, a baseline blood sample (5 mL) was collected for measurement of PTH, PO₄, ACa and Ca²⁺. Thirty minutes of treadmill running at 55%, 65% or 75% VO_{2max} commenced thereafter. Additional blood was collected after 2.5, 5, 7.5, 10, 15, 20, 25 and 30 minutes of exercise. After exercise, participants adopted a semi-recumbent position and blood was collected at 32.5, 35, 37.5, 40, 45, 50, 55, 60, 90, 120 and 180 minutes. Ca²⁺ was measured immediately but due to equipment availability Ca²⁺ was only measured in participants 5 – 10. Blood was treated and stored according to Section 3.3.2.2. Following the last blood sample, the cannula was removed and body mass measured. Participants were given 3 mL·kgBM⁻¹·h⁻¹ of water to consume throughout the trials. The timings of blood samples and exercise were identical in each trial to ensure that circadian rhythms of the metabolites were controlled for.

6.2.5 Biochemical analysis

PTH, PO₄, total calcium, albumin, Ca²⁺ and pH were measured according to Sections 3.4.2, 3.4.3 and 3.4.4.

6.2.6 Statistical analysis

As well as the analysis performed as reported in Section 3.8, Pearson's correlation coefficients were calculated for PO₄, ACa and Ca²⁺ with PTH. Cross-correlational analyses were also performed to determine the temporal relationships between PTH and PO₄, ACa and Ca²⁺. Cubic interpolation was performed to adjust for unevenly spaced data points and cross-correlational analyses were subsequently performed using R (version 3.2.2, Vienna, Austria). To determine whether one time series led another, cross-correlation functions were computed at seven lag time points for 'PEAK' (data points between baseline and peak PTH concentrations [5 minutes of recovery]), where each lag represented 3.5 minutes, and six lag time points for 'DEC' (all data points during the decrease in PTH concentrations [5 to 90 minutes of recovery]), where each lag represented 8 minutes. The cross-correlation analysis allowed for a set number of intervals, based on the sampling frequencies *i.e.* a

maximum of 20 intervals, as there were 20 blood samples taken. The number of intervals chosen was related to the total amount of time in each phase of the protocol ('PEAK' and 'DEC'). Lag times were determined by the time in 'PEAK' and 'DEC' and the number of samples, to allow for evenly distributed samples across the two time periods.

6.3 Results

6.3.1 Baseline biochemistry

Baseline PTH, PO₄, ACa and albumin were not significantly different between trials ($P=0.339$ to 0.982). Baseline Ca²⁺ at 55% VO_{2max} was significantly ($P\leq 0.05$) higher than at 65% VO_{2max} ($d=0.66$) and 75% VO_{2max} ($d=1.00$) (Table 17).

Table 17. Baseline biochemistry across all trials.

Measure	55% VO _{2max}	65% VO _{2max}	75% VO _{2max}
PTH (pmol·L ⁻¹)	2.62 ± 0.88	2.51 ± 0.50	2.63 ± 0.60
PO ₄ (mmol·L ⁻¹)	1.14 ± 0.12	1.17 ± 0.25	1.12 ± 0.16
ACa (mmol·L ⁻¹)	2.32 ± 0.11	2.30 ± 0.11	2.31 ± 0.08
Albumin (g·dL ⁻¹)	4.60 ± 0.14	4.63 ± 0.19	4.57 ± 0.22
Ca ²⁺ (mmol·L ⁻¹)	1.27 ± 0.03 ^a	1.25 ± 0.02	1.24 ± 0.01

Data are mean ± 1SD. ^a = Baseline Ca²⁺ at 55% VO_{2max} was significantly ($P\leq 0.05$) higher than at 65% and 75% VO_{2max}.

6.3.2 PTH

There was no main effect of *Intensity*, but there was a main effect of *Time* ($P\leq 0.001$) and there was a significant *Intensity* × *Time* interaction ($P\leq 0.001$). PTH concentrations decreased with the onset of exercise and were significantly lower than baseline after 5 minutes of exercise at 55% VO_{2max} (-23%; $P\leq 0.05$) and 75% VO_{2max} (-33%; $P\leq 0.001$), but not at 65% VO_{2max} (-21%; $P=0.305$) (Figure 16A all participants; Figure 17A participants 5–10). Thereafter, PTH increased, becoming significantly greater than baseline at the end of exercise (30 minutes) at 75% VO_{2max} (+52%; $P\leq 0.001$) and after 2.5 minutes of recovery at 55% VO_{2max} (+43%; $P\leq 0.001$) and 65% VO_{2max} (+52%; $P\leq 0.001$). PTH concentrations peaked after 5 minutes of recovery at 55% VO_{2max} (+73%; $P\leq 0.001$) and 75% VO_{2max} (+110%; $P\leq 0.001$), and after 7.5 minutes of recovery at 65% VO_{2max} (+76; $P\leq 0.001$). PTH concentrations then decreased, but remained significantly higher than baseline until 15 minutes into recovery at 55% VO_{2max} and until 25 minutes at 65% VO_{2max} and 75% VO_{2max}. PTH concentrations

decreased below baseline after 60 minutes of recovery in all trials (-8% to -17%). PTH concentrations were not significantly different at any time point between 55% and 65% $\text{VO}_{2\text{max}}$ trials. Exercise at 75% $\text{VO}_{2\text{max}}$ resulted in significantly higher PTH concentrations than at 55% $\text{VO}_{2\text{max}}$ at the end of exercise ($P \leq 0.001$, $d=0.90$), and at 2.5 ($P \leq 0.001$, $d=0.92$), 5 ($P \leq 0.001$, $d=0.84$), 7.5 ($P \leq 0.05$, $d=0.65$), 10 ($P \leq 0.05$, $d=0.69$) and 15 ($P \leq 0.001$, $d=0.94$) minutes into recovery, and higher than exercise at 65% $\text{VO}_{2\text{max}}$ at the end of exercise ($P \leq 0.001$, $d=0.81$), and at 2.5 ($P \leq 0.001$, $d=1.07$) and 5 ($P \leq 0.001$, $d=1.25$) minutes into recovery.

6.3.3 PO_4

There was no main effect of *Intensity*, but there was a main effect of *Time* ($P \leq 0.001$) and there was a significant *Intensity x Time* interaction ($P \leq 0.05$). PO_4 concentrations increased with the onset of exercise at all intensities, being significantly higher than baseline from 7.5 minutes to the end of exercise at 55% $\text{VO}_{2\text{max}}$ (+16%; $P \leq 0.001$), and between 5 minutes and the end of exercise at 65% $\text{VO}_{2\text{max}}$ (+22%) and 75% $\text{VO}_{2\text{max}}$ (+26%) ($P \leq 0.05$ to $P \leq 0.001$) (Figure 16B). PO_4 concentrations peaked at the end of exercise, and decreased thereafter, but remained significantly higher than baseline until 5 minutes into recovery at 55% $\text{VO}_{2\text{max}}$, 10 minutes at 65% $\text{VO}_{2\text{max}}$ and 15 minutes at 75% $\text{VO}_{2\text{max}}$. PO_4 concentrations decreased below baseline at 60 minutes of recovery and remained so until 150 minutes of recovery at 65% $\text{VO}_{2\text{max}}$ (-5 to -10%) and 75% $\text{VO}_{2\text{max}}$ (-7 to -12%) ($P \leq 0.05$ to $P \leq 0.001$). Concentrations did not decrease significantly below baseline at 55% $\text{VO}_{2\text{max}}$. Exercise at 65% $\text{VO}_{2\text{max}}$ resulted in significantly higher PO_4 concentrations than exercise at 55% $\text{VO}_{2\text{max}}$ at 10 ($P \leq 0.05$, $d=0.43$), 20 ($P \leq 0.001$, $d=0.59$) and 25 ($P \leq 0.05$, $d=0.62$) minutes of exercise.

6.3.4 ACa

There was no main effect of *Intensity*, but there was a main effect of *Time* ($P \leq 0.001$) and there was a significant *Intensity x Time* interaction ($P \leq 0.001$). ACa concentrations increased with the onset of exercise and were significantly higher than baseline between 2.5 minutes and the end of exercise at 65% $\text{VO}_{2\text{max}}$ and 75% $\text{VO}_{2\text{max}}$ (+4 to +8%; $P \leq 0.001$) (Figure 16C). ACa concentrations peaked after 20 minutes of exercise and decreased thereafter, but remained significantly higher than baseline until

5 minutes into recovery at 65%VO_{2max} and 7.5 minutes into recovery at 75%VO_{2max}. Aca concentrations decreased below baseline 10 minutes into recovery and remained so until 25 min of recovery at 55%VO_{2max} (-4%; $P\leq 0.05$). Concentrations decreased below baseline 25 minutes into recovery and remained so until 90 minutes of recovery at 65%VO_{2max} (-2%; $P\leq 0.05$). Aca concentrations did not decrease significantly below baseline at 75%VO_{2max}. Exercise at 75%VO_{2max} resulted in significantly higher Aca concentrations than exercise at 55%VO_{2max} after 20 ($P\leq 0.05$, $d=0.85$), 25 ($P\leq 0.001$, $d=1.07$) and 30 minutes of exercise ($P\leq 0.001$, $d=1.05$) and after 25 minutes of recovery ($P\leq 0.01$, $d=0.73$).

6.3.5 Albumin

There was no main effect of *Intensity*, but there was a main effect of *Time* ($P\leq 0.001$) and there was a significant *Intensity x Time* interaction ($P\leq 0.01$). Albumin concentrations increased with the onset of exercise and were higher than baseline between 7.5 minutes and the end of exercise at 65%VO_{2max} (+4%; $P\leq 0.05$) and between 5 minutes of exercise and the end of exercise at 75%VO_{2max} (+6%; $P\leq 0.05$) (Figure 16D). Albumin concentrations peaked after 20 minutes of exercise and decreased thereafter, but remained higher than baseline until 5 minutes into recovery at 75%VO_{2max} ($P\leq 0.001$). Albumin concentrations decreased below baseline 25 minutes into recovery and remained so until 90 minutes of recovery at 55%VO_{2max} (-3 to -4%; $P\leq 0.01$). Concentrations decreased below baseline 20 minutes into recovery and remained so until 90 minutes of recovery at 65%VO_{2max} (-3 to -5%; $P\leq 0.05$ to $P\leq 0.001$). Albumin concentrations did not decrease below baseline at 75%VO_{2max}. Exercise at 75%VO_{2max} resulted in significantly higher albumin concentrations than exercise at 55%VO_{2max} after 25 minutes of exercise ($P\leq 0.05$, $d=0.82$).

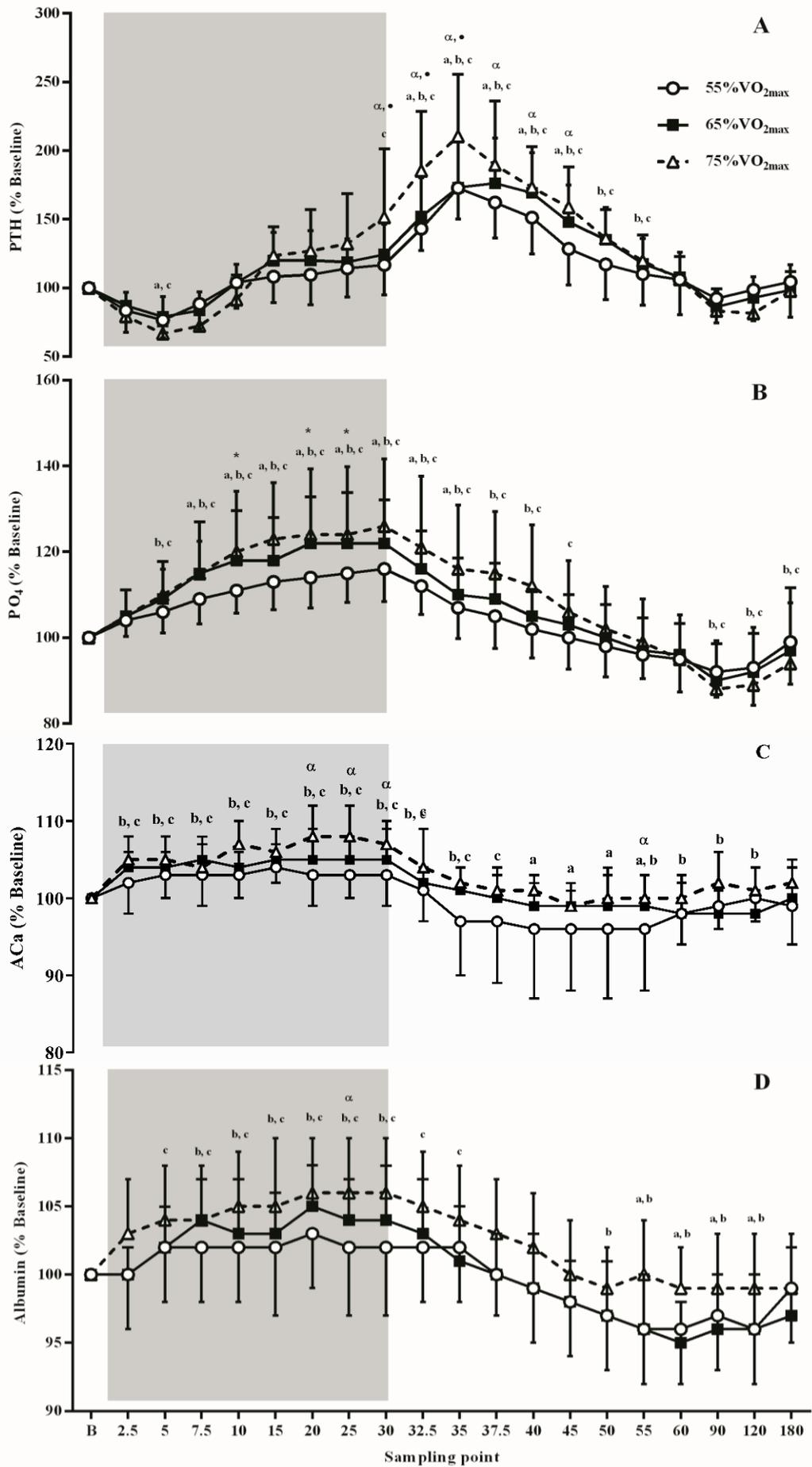


Figure 16. The percentage change in baseline concentrations of PTH (A), PO₄ (B), ACa (C) and albumin (D) for all participants with 30 minutes of treadmill running at 55%VO_{2max} (open circles), 65%VO_{2max} (filled squares), 75%VO_{2max} (open triangles). Grey box denotes exercise. Data are mean ± 1SD. ^a different ($P \leq 0.05$) from baseline (55%VO_{2max}) ^b different ($P \leq 0.05$) from baseline (65%VO_{2max}), ^c different ($P \leq 0.05$) from baseline (75%VO_{2max}). * 55%VO_{2max} different ($P \leq 0.05$) from 65%VO_{2max}, ^a 55%VO_{2max} different ($P \leq 0.05$) from 75%VO_{2max}, [•] 65%VO_{2max} different ($P \leq 0.05$) from 75%VO_{2max}.

6.3.6 Ca²⁺

There was no main effect of *Intensity*, but there was a main effect of *Time* ($P \leq 0.001$) and there was a significant *Intensity x Time* interaction ($P \leq 0.001$). At 55%VO_{2max}, Ca²⁺ concentrations decreased after 10 minutes of exercise, being significantly below baseline between 25 minutes and the end of exercise (Figure 17B) (-2%; $P \leq 0.001$). Ca²⁺ concentrations continued to decrease into recovery, remaining significantly below baseline until 90 minutes of recovery (-2 to -6%; $P \leq 0.001$). At 65%VO_{2max} and 75%VO_{2max} Ca²⁺ concentrations increased with the onset of exercise and were significantly higher than baseline between 2.5 and 10 minutes of exercise at 65%VO_{2max} (+2 to +3%; $P \leq 0.001$) and between 2.5 and 7.5 minutes at 75%VO_{2max} (+2 to +3%; $P \leq 0.001$). Thereafter, Ca²⁺ concentrations decreased and were significantly below baseline between 2.5 and 30 minutes of recovery at 65%VO_{2max} (-3 to -4%; $P \leq 0.05$ to $P \leq 0.001$) and 75%VO_{2max} (-3 to -4%; $P \leq 0.001$). There were no significant differences between the three trials at any time point other than at baseline (Table 17), which created the significant *Intensity x Time* interaction.

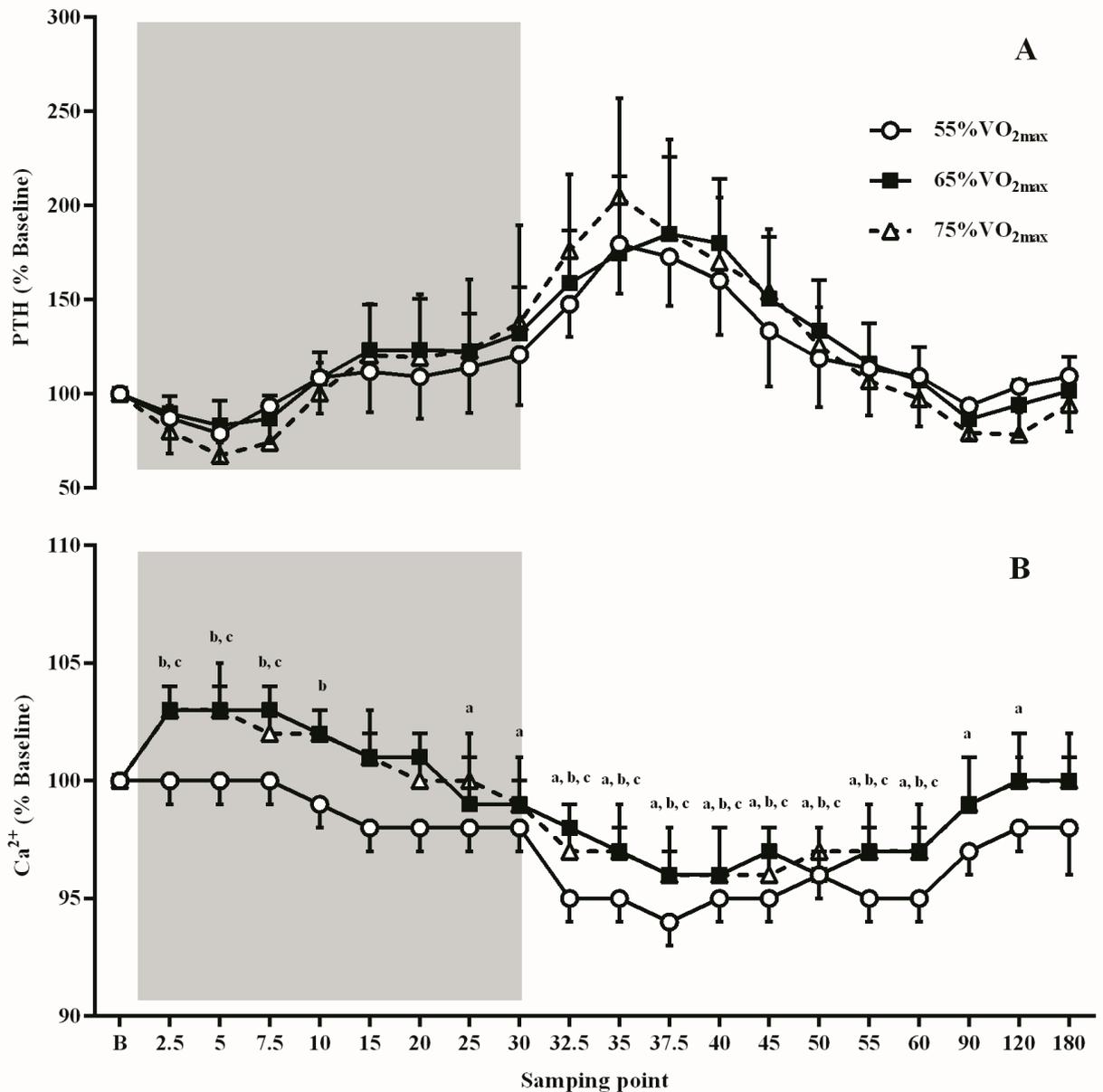


Figure 17. The percentage change in baseline concentrations of PTH (A) and Ca²⁺ (B) for participants 5 – 10 with 30 minutes of treadmill running at 55% VO_{2max} (open circles), 65% VO_{2max} (filled squares), 75% VO_{2max} (open triangles). Grey box denotes exercise. Data are mean ± 1SD. ^a different ($P \leq 0.05$) from baseline (55% VO_{2max}) ^b different ($P \leq 0.05$) from baseline (65% VO_{2max}), ^c different ($P \leq 0.05$) from baseline (75% VO_{2max}). * 55% VO_{2max} different ($P \leq 0.05$) from 65% VO_{2max}, ^a 55% VO_{2max} different ($P \leq 0.05$) from 75% VO_{2max}, • 65% VO_{2max} different ($P \leq 0.05$) from 75% VO_{2max}. Statistical analysis not reported or denoted for the PTH response in participants 5 – 10; data plotted for the comparison with Ca²⁺ only.

6.3.7 pH

There were no significant changes in pH throughout exercise at any exercise intensity and pH at baseline and the end of exercise was not significantly different between trials (Table 18).

Table 18. pH values at baseline and at the end of exercise.

Exercise intensity	pH	
	Baseline	End of exercise
55% VO _{2max}	7.343 ± 0.020	7.412 ± 0.044
65% VO _{2max}	7.355 ± 0.030	7.408 ± 0.039
75% VO _{2max}	7.343 ± 0.041	7.396 ± 0.026

Data are mean ± 1SD.

6.3.8 Correlation analyses

Changes in PTH were not significantly correlated with changes in PO₄ or ACa in any trial. Across all data points PTH was significantly ($P \leq 0.001$) negatively correlated with Ca²⁺ at all intensities (Table 19).

Table 19. Pearson's correlation coefficient values for changes in PTH, with changes in PO₄, ACa and Ca²⁺.

Exercise intensity	<i>r</i> value		
	PO ₄	ACa	Ca ²⁺
55% VO _{2max}	0.175	-0.216	-0.739 ^a
65% VO _{2max}	0.215	-0.174	-0.769 ^a
75% VO _{2max}	0.416	0.089	-0.790 ^a

^a Significant correlation with PTH ($P \leq 0.001$).

Across PEAK data points, PO₄ was correlated with PTH at all exercise intensities ($r = 0.661$ to 0.772) (Table 20) when the PTH series was lagged by 1 time point (3.5 minutes) behind the PO₄ series,

suggesting that increases in PO₄ precede increases in PTH by 3.5 minutes. Ca²⁺ was most strongly correlated with PTH at all exercise intensities ($r = -0.902$ to -0.950) when there was no time lag, suggesting that increases in PTH occur within 3.5 minutes of a decrease in Ca²⁺ (Table 20).

Across DEC data points, PO₄, ACa and Ca²⁺ were correlated with PTH at all exercise intensities. PO₄ was most strongly correlated with PTH at all exercise intensities ($r = 0.987$ to 0.995) (Table 20) when there was no time lag, suggesting that decreases in PTH occur within 8 minutes of a decrease in PO₄.

Table 20. Maximum cross-correlation values and corresponding lag times for PTH with PO₄, ACa and Ca²⁺.

Exercise intensity	PO ₄		ACa		Ca ²⁺	
	Time lag	<i>r</i> value	Time lag	<i>r</i> value	Time lag	<i>r</i> value
<i>PEAK data points (baseline to 5 minutes of recovery)</i>						
55% VO _{2max}	-1	0.661	0	-0.599	0	-0.902
65% VO _{2max}	-1	0.677	-2	0.555	0	-0.936
75% VO _{2max}	-1	0.772	-2	0.659	0	-0.950
<i>DEC data points (5 to 90 minutes of recovery)</i>						
55% VO _{2max}	0	0.995	0	0.690	+1	-0.794
65% VO _{2max}	0	0.987	0	0.858	0	-0.856
75% VO _{2max}	0	0.994	0	0.798	+1	-0.817

6.4 Discussion

The novel findings from this study are: 1) changes in PTH, PO₄, ACa and Ca²⁺ occur within 2.5 minutes of the onset of exercise; 2) there is an initial decrease in PTH concentrations at the start of exercise that coincides with a significant increase in Ca²⁺ concentrations at the two higher exercise intensities; 3) peak PTH concentrations occur within 5 – 7.5 minutes of recovery; 4) increases in PO₄ precede increases in PTH; 5) decreases in Ca²⁺ precede increases in PTH; 6) post-exercise decreases in PTH concentrations are preceded by decreases in PO₄.

The pattern of change in PTH in this study is comparable to previous studies, with PTH concentrations increasing during exercise (Scott *et al.*, 2010; Scott *et al.*, 2011; Scott *et al.*, 2012; Scott *et al.*, 2013; Scott *et al.*, 2014) and peaking in the first minutes of recovery (Maimoun *et al.*, 2006). The pattern of change in PTH was similar across the three exercise intensities, with an initial decrease from baseline to 5 minutes of exercise. This is the first study to observe this initial response in PTH, due to the higher temporal frequency of blood sampling at the start of exercise compared with previous studies. This response requires verification from further studies and the use of even more frequent sampling. The lack of a resting control group in the present study means that we cannot confirm whether this is a characteristic physiological response to the onset of exercise or whether this reflects the circadian rhythm of PTH at the time of sampling. The nadir in PTH occurs between 08:00 and 10:00 (Jubiz *et al.*, 1972; Logue *et al.*, 1989; Fraser *et al.*, 1994; Fuleihan *et al.*, 1997) and our baseline blood was taken at 08:55, with exercise commencing at 09:02. If the initial decrease in PTH were due to the circadian rhythm, however, it would be expected that the decrease would have lasted longer than 5 minutes into exercise. Additionally, a decrease of 33% from baseline, followed by a rapid reversal in the direction of change, as shown here, has not been reported in circadian studies.

Peak PTH concentrations have previously been shown to occur 15 minutes after exercise (Maimoun *et al.*, 2006), due to a lower sampling frequency, but the results of the present study show that the peak in PTH after exercise occurs with 5 – 7.5 minutes of recovery (+73 to +110% from baseline). This peak is also transient; PTH concentrations immediately start to decrease after reaching peak

concentrations, and these transient spikes are anabolic for bone (Frolik *et al.*, 2003), and result in net bone gain (Dempster *et al.*, 1993). Our identification of peak PTH concentrations 5 – 7.5 minutes after exercise could be utilised to improve bone health in individuals at risk of impact fractures, stress fractures or poor bone health, such as endurance athletes. This could involve the development of a training session involving bouts of running sufficient to cause a spike in PTH concentrations, followed by rest periods to ensure that the spike is transitory. Endurance athletes often train for prolonged periods and cover large distances, for example, distance runners can run over 200 km·week⁻¹ (Billat *et al.*, 2001). This type of training involves low-strain repetitive loading, therefore these athletes may benefit from performing shorter, higher intensity running sessions if they do not already incorporate these into training schedules.

Cross-correlations suggested that PTH secretion during exercise and recovery is controlled by a combination of changes in Ca²⁺ and PO₄. Ca²⁺ is not routinely measured due to analytical difficulties; consequently ACa is estimated as a surrogate and has been shown clinically to be a reliable indicator of calcium metabolism at rest (White *et al.*, 2010). We have shown different responses to exercise and recovery between ACa and Ca²⁺ and also different relationships with PTH; Ca²⁺ concentrations were correlated with PTH, whereas ACa was not. Albumin changes taking place during exercise will have a greater effect on the ACa estimation compared to the small effect that can occur on the Ca²⁺ measurement. There were no significant changes in pH throughout exercise so changes in pH were not likely to be sufficient to have a major effect on the Ca²⁺ measurement by the blood gas analyser.

The results support previous data (Barry and Khort, 2007; Scott *et al.*, 2010; Scott *et al.*, 2011; Scott *et al.*, 2012; Scott *et al.*, 2013; Scott *et al.*, 2014) suggesting that changes in ACa do not explain the changes in PTH or regulation of PTH during exercise, because, as PTH is increasing, ACa either also increases (Scott *et al.*, 2010; Scott *et al.*, 2011) or is unchanged (Barry and Khort, 2007; Scott *et al.*, 2012; Scott *et al.*, 2013). Scott *et al.* (2013) argued that because both PTH and ACa were increased after 20 minutes of exercise, a decrease in Ca²⁺ could have occurred in the first few minutes of exercise, stimulating the secretion of PTH and causing serum Ca²⁺ concentrations to increase as a result of PTH-stimulated bone resorption and Ca²⁺ liberation. However, through frequent sampling,

we have shown that A_{Ca} and Ca²⁺, at 65% and 75% VO_{2max}, increase within 2.5 minutes of exercise, with A_{Ca} increasing and Ca²⁺ decreasing thereafter. Dermal calcium losses could contribute to this decrease in Ca²⁺ during exercise (Chapters 4 and 5), but this is a hypothesis that requires investigation. Although it is well established that PTH responds rapidly to a reduction in Ca²⁺ at rest (Brown, 1983; Brown, 2000), this is the first study to show that this rapid response also occurs during exercise. The lack of an initial increase in Ca²⁺ at 55% VO_{2max} is surprising and the reason for this is currently unknown. The strong negative correlation of PTH and Ca²⁺ during exercise, at all three intensities, with a 0 time lag ($r = -0.902$ to -0.950), suggests that as Ca²⁺ decreases, PTH increases within 3.5 minutes. This negative cross-correlation supports the findings of Bouassida *et al.* (2003) who showed that as Ca²⁺ decreased during 42 minutes of running, PTH increased.

The significantly higher baseline concentration of Ca²⁺ at 55% VO_{2max} compared to 65% and 75% VO_{2max} is surprising and possible reasons for this difference were explored. There were no spurious results and the timing of the sampling was identical in all trials within participants, so the difference is unlikely to be due to a circadian change. All trials were completed in a randomised (Latin Squared Design) and counterbalanced order, so the difference is unlikely to be due to changes in vitamin D status or other environmental factors. The quality control and calibration reports of the blood gas analyser used to measure Ca²⁺ were checked and there were no issues reported. Participants did not systematically change behaviour between trials, *i.e.* there was no reported change in activity levels or dietary changes. Participants reported that they followed the same diet and restrained from physical activity before each trial. Despite this, the magnitude of the difference is small; 1.27 mmol·L⁻¹ compared to 1.25 mmol·L⁻¹ and 1.24 mmol·L⁻¹, with small standard deviations. This reflects the tightly regulated control of serum calcium concentrations. Taking this information into account, there is no known reason for the different baseline concentration between trials, but is something that needs to be investigated in future studies, as this higher initial Ca²⁺ concentration in the 55% VO_{2max} trial could be the reason for the lack of an initial increase in Ca²⁺ at the start of exercise.

The strong negative cross-correlation of PTH and Ca²⁺ suggest that Ca²⁺ may control PTH secretion during exercise. The reasons for the initial increase in Ca²⁺ at the start of exercise in the two higher

exercise intensities are unknown. This is important because it is likely to explain the novel finding of decreased PTH concentrations with the onset of exercise. It could have been related to exercise-induced acidosis occurring in the first few minutes of exercise, before aerobic metabolism stabilises (Skinner and McLellan, 1980; Bogdanis *et al.*, 1996). This can increase Ca^{2+} concentrations (Beck and Webster, 1976) but have minimal effects on ACa. Blood pH did not, however, decrease significantly during exercise, suggesting that exercise-induced acidosis was not the reason for the initial increase in Ca^{2+} . The initial increase could be from calcium being released from other binding proteins such as transferrin (Scott and Bradwell, 1983) or calcium dissociating from PO_4 (Walser, 1961; Chertow *et al.*, 1999). The initial increase could be related to an increase in intestinal calcium absorption as blood flow around the body increases due to increased heart rate and vasodilation, which is then restricted as exercise continues and blood flow is redirected to the muscles and skin away from organs such as the intestine and stomach (Savard *et al.*, 1988; Saltin *et al.*, 1998). Other additional mechanisms that might explain the initial increase in Ca^{2+} in the two higher intensities could be related to the higher frequency of cross-bridge cycling, where there may be greater concentrations of Ca^{2+} in the sarcoplasm and greater levels of dissociation and reuptake in the muscle and greater calcium efflux out of the muscle (Allen *et al.*, 1995; Fitts, 2008). Further, calcium is involved in mechanotransduction; the most obvious mechanism is the large transient increase in calcium that occurs with an action potential and active force generation. The accompanying mechanical deformation of the cell may allow calcium influx and efflux through various channels (Burkholder, 2007). Further mechanistic studies are needed to identify why this initial increase occurs.

Changes in systemic PO_4 can influence PTH secretion, with Ahmad *et al.* (2003) showing that circadian changes in PO_4 precede changes in PTH. During the increase in PTH shown in the present study, PO_4 and PTH were most strongly positively cross-correlated at -1 time lag, suggesting that increases in PO_4 precede increases in PTH by less than 3.5 minutes. This cross-correlation was not as strong, however, as the cross-correlation between Ca^{2+} and PTH, which might indicate that both PO_4 and Ca^{2+} are influential during the increase in PTH. Our data do not fully support that the exercise-induced increases in PTH are driven solely by increased PO_4 , as PO_4 increased with the

onset of exercise, despite the initial decrease in PTH. The increase in PO₄ might reflect release of PO₄ from PTH-induced bone resorption (Estepa *et al.*, 1999; Ahmad *et al.*, 2003; Scott *et al.*, 2010) towards the end of exercise, or that PO₄ is being released from muscle tissue (Forrester and Lind, 1969; Dobson *et al.*, 1971). Taken together, these results suggest that Ca²⁺ is the stronger driver of PTH secretion and synthesis at the onset of exercise, although it is possible that the degree of association/dissociation between Ca²⁺ and PO₄ varies during exercise, meaning that PTH regulation might change accordingly.

With the decrease in PTH during recovery, the strongest positive cross-correlation between PO₄ and PTH occurred at a 0 time lag, suggesting that PTH decreased within 8 minutes of a decrease in PO₄. These findings support Scott *et al.* (Scott *et al.*, 2010; Scott *et al.*, 2012; Scott *et al.*, 2013; Scott *et al.*, 2014), who showed that PO₄ followed the same responses as PTH after exercise. If the decrease in PTH during recovery is explained by renal clearance (Bouassida *et al.*, 2003), the strong cross-correlation may suggest that PO₄ is driving PTH clearance and over-riding Ca²⁺ regulation in recovery. Alternatively, the elevated PTH concentrations could be enhancing renal PO₄ excretion and causing a subsequent decrease in circulating PO₄ (Silver *et al.*, 2000).

Small reductions in vitamin D concentrations can contribute to an increase in PTH, because 1,25-dihydroxyvitamin D regulates the active transport of calcium and PO₄ absorption in the small intestine (Heaney and Barger-Lux, 1985; Heaney *et al.*, 2003; Cashman, 2007). This is important because vitamin D deficiencies are common in athletes, particularly during winter months, and low serum vitamin D concentrations have been associated with stress fracture injuries (Lappe *et al.*, 2008; Nieves *et al.*, 2010; Sonnevile *et al.*, 2012; Close *et al.*, 2013; Wolman *et al.*, 2013; Miller *et al.*, 2016). Vitamin D status was not measured in this study so we cannot determine whether a change in vitamin D status occurred throughout the study. Despite this, the three trials were completed within one month for each participant and the order of trials was randomised, therefore although changes in vitamin D concentrations could have occurred, it is unlikely to have influenced the results of the present study. Moreover, the results of the studies reported in Chapters 4 and 5 showed that elite

triathletes have sufficient vitamin D concentrations in both the winter and spring and with or without supplementation.

In conclusion, at the onset of exercise, PTH transiently decreases and then increases as exercise continues, peaking in the first minutes of recovery, before decreasing below the baseline concentration during ongoing recovery. Changes in Ca^{2+} and PO_4 occur in close temporal relation to changes in PTH. Cross-correlational analysis suggests that PTH secretion during exercise and recovery is controlled by a combination of changes in Ca^{2+} and PO_4 and that the mechanism might be different during exercise and recovery. A Ca may not be a suitable surrogate for Ca^{2+} when investigating the rapid response to exercise, since A Ca concentrations do not reflect temporal PTH responses or correlate strongly with PTH. Since we now have a more detailed understanding of the mechanism controlling PTH during exercise and recovery, future studies could investigate the response to calcium supplementation before or during training sessions in elite athletes. This may prevent excessive increases in PTH and bone resorption that may be caused, in part, by high rates of dermal calcium loss during intense training. Furthermore, the hypothesis that dermal calcium losses contribute to decreases in Ca^{2+} concentrations during exercise needs to be proven and the difference in the PTH response to cycling and running should also be investigated.

CHAPTER 7: INVESTIGATING THE EFFECT OF POST-EXERCISE CARBOHYDRATE AND PROTEIN INGESTION ON BONE METABOLISM IN TRAINED ENDURANCE ATHLETES

This study has been submitted to the Journal of Medicine and Science in Sport and Exercise and has been presented at the Bone Research Society Annual Meeting where it was awarded 'Best Oral Poster':

Townsend, R., Elliott-Sale, K.J., Currell, K., Tang, J., Fraser, W.D. and Sale, C. (2016). The Effect of Post-Exercise Carbohydrate and Protein Ingestion on Bone Metabolism.

Townsend, R., Elliott-Sale, K.J., Currell, K., Fraser, W.D. and Sale, C. Ingestion of Carbohydrate and Protein Immediately After an Exhaustive Run Suppresses Bone Resorption and Increases Bone Formation in Trained Male Endurance Runners. *Bone Research Society Annual Meeting, June 2016, Liverpool, UK.*

7.1 Introduction

The results of the studies reported in Chapters 4 and 5 showed that elite triathletes struggle to match energy intakes with energy expenditures. After feedback of the results to the British triathletes, a commonly reported reason for energy intakes being inadequate is the minimal time between training sessions, leaving insufficient time for the triathletes to consume the necessary energy and nutrients throughout the day. It is difficult for triathletes to sufficiently increase overall energy intake and match this with the energy demands of such extensive training schedules. It is therefore necessary for other feeding practices and nutritional interventions to be implemented surrounding training sessions. These feeding practices should be practical and realistic for elite triathletes to adhere to, but should work effectively to prevent an increase in bone resorption and an imbalance in bone turnover that is often shown after an acute bout of intense exercise (Scott *et al.*, 2010). As the results of the studies reported in Chapters 4 and 5 showed that elite triathletes have high bone turnover at different phases of the season, maintaining a balanced bone turnover and anabolic conditions for bone during and after individual training sessions is important, given that accelerated bone remodelling can cause microdamage accumulation and has been implicated in the formation of stress fracture injuries (Parfit, 1982; Schaffler *et al.*, 1990; Martin, 1992; Burr, 2002; Schaffler, 2003; Warden *et al.*, 2006).

Nutritional status and dietary practices are vitally important for elite athletes as they can influence both acute bone turnover and long-term bone health (Babraj *et al.*, 2005; Walsh and Henriksen, 2010) and acute feeding influences the diurnal rhythm of bone turnover markers at rest (Schlemmer and Hassager, 1999). Feeding of a mixed nutrient meal suppresses all markers of bone turnover (Clowes *et al.*, 2002a) and feeding of individual nutrients; glucose, fat, protein and calcium, also suppresses bone resorption at rest (Blumsohn *et al.*, 1994a; Bjarnson *et al.*, 2002; Clowes *et al.*, 2003; Henriksen *et al.*, 2003). Previous studies have only investigated the effect of nutrient ingestion on bone turnover markers in resting, non-athletic participants, who have not performed any prior exercise. It is therefore not known whether there is a similar suppressive effect of nutrient ingestion on bone resorption, after exercise in athletic individuals.

Pre-exercise feeding has been investigated as a potential means for attenuating the bone resorption response to exercise. Scott *et al.* (2012) showed that feeding a mixed nutrient breakfast prior to exercise had no effect on post-exercise β -CTX concentrations compared to fasting and there were no changes in markers of bone formation. This implies that the mechanical loading experienced during exercise over-rides any responses caused by pre-exercise feeding, Scott *et al.* (2012) also suggested that the stimulatory effect of PTH on β -CTX may override the effect of pre-exercise feeding. Furthermore, eating in close proximity to exercise can cause gastrointestinal discomfort during the training session and athletes are often concerned that this will impair performance (Haakonssen *et al.*, 2012), therefore other exercise feeding practices and the subsequent PTH response require investigation.

Sale *et al.* (2015) showed that CHO feeding during exercise attenuated β -CTX and PINP responses in the hours following exercise, indicating an acute effect of CHO feeding on bone turnover. Feeding during intense running might not, however, be well tolerated by athletes and could be restricted by opportunity and practicality (Rehrer *et al.*, 1992; Peters *et al.*, 1999; Peters *et al.*, 2001; Pfeiffer *et al.*, 2009; Pfeiffer *et al.*, 2012). Post-exercise feeding provides a practical opportunity to ingest multiple nutrients and in sufficient amounts, thus allowing athletes to reach other sports nutrition goals, such as aiding muscle glycogen resynthesis, protein synthesis and maintaining adequate hydration status (Jentjens and Jeukendrup, 2003; Tipton *et al.*, 2004), without the restrictions of gastrointestinal discomfort. Simple CHO and PRO are not likely to cause gastrointestinal complaints as they contain little fibre or fat, which means that digestion is quick (Rehrer *et al.*, 1992; Jenkins *et al.*, 2009). Post-exercise feeding also allows for investigation of the bone turnover response to acute feeding without the confounding effect of subsequent mechanical loading.

It is not known whether the acute bone turnover response to post-exercise feeding is the same as at rest and whether this varies with different timings of post-exercise nutrient ingestion. The aim of this study was to investigate the effect of feeding carbohydrate and protein (CHO+PRO) immediately or 2 h after a prolonged intense running bout, on the bone turnover response in trained endurance

runners and triathletes. Markers associated with exercise and bone were also measured to explore possible mediating and mechanistic factors.

7.2 Methodology

7.2.1 Participants

10 men ([mean \pm 1SD] age 28 ± 6 y, height 1.74 ± 0.05 m, body mass 69.7 ± 6.3 kg, VO_{2max} 63.0 ± 5.0 mL \cdot kg $BM^{-1}\cdot$ min $^{-1}$, weekly running distance 49.9 ± 12.5 km) completed this study that was approved by the Ethical Advisory Committee at Nottingham Trent University (Application number 348). All participants were trained endurance runners or triathletes who had been competing and training consistently for a minimum of 2 years in 10,000 m, half marathon, marathon or ultra-distance races, without a significant break. Participants had recorded at least one of the following times in the past 2 years; ≤ 35 minutes for 10,000 m, $\leq 1:25:00$ for half marathon or $\leq 3:00:00$ for marathon. Participants were recruited at local running and triathlon clubs and local races. Participants were non-smokers, had not suffered a fracture in the last 12 months, were free from musculoskeletal injury and did not suffer from any condition known to affect bone metabolism. Compliance with these inclusion criteria was confirmed in the initial visit to the laboratory, where health screening was completed and written informed consent provided.

7.2.2 Experimental design

This was a randomised (Latin Square Design), counterbalanced, placebo-controlled and single-blinded, crossover study. Participants completed a preliminary visit for habituation with trial procedures and measurement of VO_{2max} . Participants then completed three, four-day experimental trials, each separated by 1 week. On days 1 and 2, participants refrained from all exercise and followed a prescribed diet. On day 3, participants performed a bout of treadmill running, at a speed equal to 75% of their previously determined VO_{2max} , until volitional exhaustion. Blood samples (20 mL) were collected before exercise, immediately after exercise and every hour after exercise for four hours. On day 4, participants returned to the laboratory for a fasted follow-up blood sample.

The three trials consisted of; i) a placebo (PLA) control trial, where the PLA solution was ingested both immediately and 2 h post-exercise, ii) an immediate feeding (IF) trial, where the CHO+PRO solution was ingested immediately post-exercise and the PLA solution 2 h post-exercise, and iii) a

delayed feeding (DF) trial where the PLA solution was ingested immediately post-exercise and the CHO+PRO solution 2 h post-exercise. In the PLA trial, the CHO+PRO solution was ingested after the final blood sample to ensure that the energy content and the composition of the diet was identical between trials. This meant that a final PLA solution also needed to be ingested in the IF and DF trials to ensure participant blinding to the trial conditions (Figure 18).

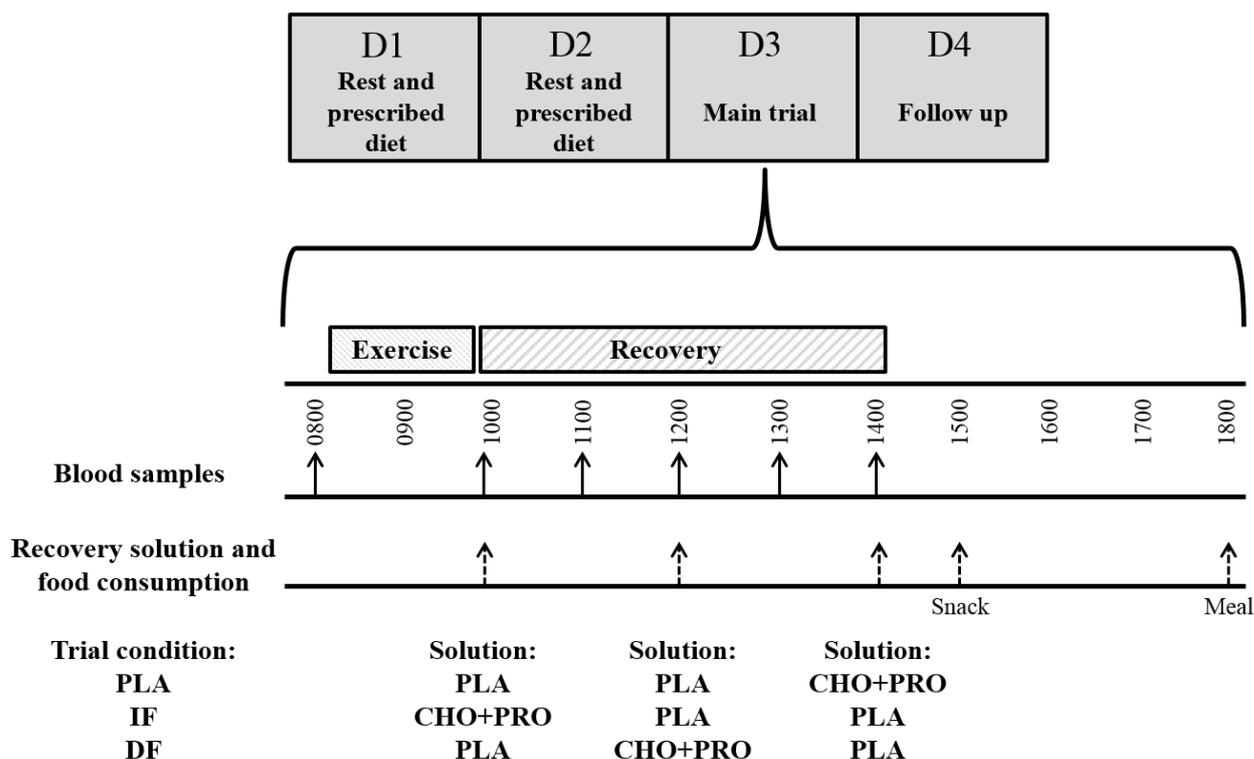


Figure 18. Experimental protocol. Exercise was treadmill running at 75% VO_{2max} , followed by 4 hours of rested recovery. PLA = Placebo trial, IF = Immediate feeding trial and DF = Delayed feeding trial. Participants departed from the laboratory at the end of the recovery period. Solid vertical arrows denote blood samples. Dashed vertical arrows denote recovery solution and food consumption.

7.2.3 Assessment of VO_{2max}

Participants performed an incremental treadmill test to determine lactate threshold, followed by a ramp test to determine VO_{2max} (Section 3.2.1). Level running velocities corresponding to 75% VO_{2max} ($13.0 \pm 0.8 \text{ km}\cdot\text{h}^{-1}$) were calculated based on the regression of VO_2 and velocity.

7.2.4 Experimental dietary provision

Participants completed a three-day food diary for the measurement of habitual energy intake and macronutrient composition. A diet consisting of 55% CHO, 30% fat and 15% PRO, and isocaloric with habitual diets was designed using dietary analysis software (Nutritics, Dublin, Ireland) (Section 3.6), for each participant to consume on days 1 and 2 of each trial (Appendix I). Participants provided their own food but were given written and verbal instructions for the preparation of meals, including timings of meals and snacks. Any deviations from prescribed diets were confirmed verbally on day 3 and recorded.

7.2.5 Experimental trial procedure

Participants were asked to maintain their habitual training and record this throughout the study to help maintain consistency across trials. Participants refrained from all exercise on days 1 and 2. Participants arrived at the laboratory on day 3 at 08:30, after fasting from 20:00 the previous evening and consuming 500 mL of water upon awakening. Shortly after arriving, participants collected a urine sample for the measurement of urine osmolality (Section 3.7), body mass was measured and the first 20 mL blood sample was taken via venepuncture after 10 minutes of semi-recumbent rest (Section 3.3.2.2).

Participants then ran to volitional exhaustion at 75% $\text{VO}_{2\text{max}}$, which was preceded by a 5-minute warm-up and volitional stretching. At exhaustion a cannula was inserted into a prominent forearm vein (Section 3.3.2.3), which was kept patent by flushing with saline, a second 20 mL blood sample was taken, with further blood samples taken at 1, 2, 3 and 4 h into recovery. Exact times of exercise commencement, time to exhaustion and blood samples were recorded and were repeated exactly in each trial within-participants to reduce the impact of circadian variations on the results. Due to differences in individual run times to exhaustion between participants, post-exercise blood sample timings vary between participants, but were controlled for within-participants. The baseline blood sample was taken at 08:40 and exercise commenced at 08:50, the blood sample at exhaustion was taken at 10:10 \pm 13 min and blood samples 1 – 4 hours post-exercise were taken at 11:10 \pm 13 min, 12:10 \pm 13 min, 13:10 \pm 13 min and 14:10 \pm 13 min.

Depending on the trial, participants were given either the CHO+PRO or PLA solution to consume immediately after exhaustion. Two and four hours after exhaustion participants were given further solutions to consume. After the final solution was consumed, participants were provided with food and were free to leave the laboratory. Participants consumed a snack at 15:00 and an evening meal at 18:00 and then remained fasted from 20:00 until the next morning. On day 4 participants arrived in the laboratory at 08:30 after consuming 500 mL of water upon awakening and a final 20 mL blood sample was taken.

7.2.6 Recovery solutions and evening meal composition

The CHO+PRO solution contained 1.5 g·kgBM⁻¹ of CHO (dextrose) and 0.5 g·kgBM⁻¹ of PRO (unflavoured whey isolate) that was made up to a 12.5% CHO solution with water. The whey isolate and dextrose mix was tested for banned substances by LGC Supplement Screening (Cambridgeshire, UK), participants were made aware of this and were asked to sign a supplement disclaimer (Appendix J and K). Preliminary testing ensured that the PLA solution was taste matched to the CHO+PRO solution using artificial sweetener and flavouring; it consisted of 12 ml·kgBM⁻¹ of water, making this the same volume as the CHO+PRO solution (Appendix L). Participants were blinded to the solutions that they were consuming throughout trials. The total volume of fluid consumed in the three recovery solutions was 2,509 ± 227 mL.

On day 3 the overall diet composition was 2,000 kcal, 55% CHO, 30% fat and 15% PRO. The recovery solution contained approximately 500 kcal depending on individual body mass, therefore the snack and evening meal contained approximately 1,500 kcal (Appendix M). Deviations from prescribed diets were confirmed verbally on day 4 and recorded. Participants were allowed to ingest plain water on an *ad libitum* basis throughout the recovery periods, although none of the participants did this during any trial.

7.2.7 Treatment and storage of blood samples

Blood was treated and stored according to Section 3.3.2.2.

7.2.8 Biochemical analysis

β -CTX, P1NP PTH, PO₄, total calcium, albumin and Ca²⁺ were measured according to Sections 3.4.2, 3.4.3 and 3.4.4.

7.2.9 Statistical analysis

Statistical analysis was performed as reported in Section 3.8. The study sample size was calculated to detect changes in β -CTX from pre- to post-exhaustive exercise, with 85% power at an alpha level of $P \leq 0.05$, based on the study by Scott *et al.* (2010). Normality and homogeneity were achieved following log transformations for PO₄ data.

To give a numerical value to the difference between bone formation and bone resorption, the bone turnover marker ratio was calculated using the following equation: $[P1NP]/([\beta\text{-CTX}] * 100)$, with a value above 1 indicating that bone turnover favours bone formation and a value below 1 indicating that bone turnover favours bone resorption. This equation was used in Lombardi *et al.* (2012) but for different bone turnover markers.

7.3 Results

7.3.1 Baseline biochemistry

Baseline concentrations of β -CTX, P1NP, PTH, ACa, albumin, total calcium, Ca^{2+} and PO_4 were not different between trials ($P=0.619$ to 0.999) (Table 21).

Table 21. Baseline biochemistry for all variables.

Baseline concentrations			
	PLA	IF	DF
β -CTX ($\text{ng}\cdot\text{mL}^{-1}$)	0.58 ± 0.27	0.52 ± 0.21	0.54 ± 0.28
P1NP ($\text{ng}\cdot\text{mL}^{-1}$)	64.92 ± 32.92	64.35 ± 30.75	66.42 ± 32.04
PTH ($\text{p}\cdot\text{mol}^{-1}$)	2.64 ± 0.76	2.69 ± 0.59	2.77 ± 0.72
ACa ($\text{mmol}\cdot\text{L}^{-1}$)	2.43 ± 0.10	2.44 ± 0.09	2.44 ± 0.07
Albumin ($\text{g}\cdot\text{dL}^{-1}$)	4.83 ± 0.15	4.85 ± 0.18	4.82 ± 0.18
Total calcium ($\text{mmol}\cdot\text{L}^{-1}$)	2.47 ± 0.10	2.48 ± 0.09	2.48 ± 0.07
Ca^{2+} ($\text{mmol}\cdot\text{L}^{-1}$)	1.26 ± 0.03	1.25 ± 0.03	1.26 ± 0.03
PO_4 ($\text{mmol}\cdot\text{L}^{-1}$)	1.11 ± 0.15	1.13 ± 0.16	1.17 ± 0.14

Data are mean \pm 1SD.

7.3.2 Habitual diet and experimental dietary provision

There were no significant differences between the diets prescribed for days 1, 2 and 3 of each trial and the diets that were actually consumed by participants, for overall energy content or macronutrient composition. Participants' habitual diets were not different from the diet provided on day 3 of trials, for overall energy content, CHO content, fat content and calcium content ($P=0.101$ to 0.523). However, PRO content was significantly higher in the habitual diets compared to the experimental trial diet ($P=0.049$) (Table 22).

Table 22. Habitual diet and experimental diet contents.

	Habitual diet + 48 h lead in	Experimental diet (D3)
Energy (kcal)	2,318 ± 673	2,008 ± 11
CHO (%)	51 ± 9	55 ± 0
PRO (%)	22 ± 10 ^a	15 ± 0 ^a
Fat (%)	27 ± 5	30 ± 0
Calcium (mg)	1,012 ± 302	971 ± 258

Data are mean ± 1SD. ^a PRO content of the habitual diet was significantly higher than PRO content of the experimental diet on day 3 ($P=0.049$).

7.3.3 Hydration status

Pre-exercise urine osmolality was not different between trials ($P=0.721$) (Table 22).

Table 23. Pre-exercise urine osmolality.

Urine osmolality (mOsm·L ⁻¹)		
PLA	IF	DF
493 ± 264	396 ± 274	463 ± 279

Data are mean ± 1SD.

7.3.4 Exercise variables

The average time to exhaustion (exercise duration) was 01:15:00 ± 00:13:00, with a range of 01:06:00 to 01:37:00. There was a significant decrease in body mass from pre-exercise (69.4 ± 6.1 kg) to post-exercise (68.9 ± 5.9 kg) ($P=0.001$).

7.3.5 Bone turnover markers

7.3.5.1 β-CTX

There was a significant main effect of *Trial* ($P\leq 0.001$), *Time* ($P\leq 0.001$) and a significant *Trial* × *Time* interaction ($P\leq 0.001$) for β-CTX. β-CTX concentrations were increased from baseline by the

end of exercise in all trials (+8 to +12%). In the PLA trial, β -CTX concentrations remained increased above baseline at 1 h post-exercise (+7%), before decreasing thereafter, being significantly lower than baseline concentrations 3 and 4 h post-exercise (-31 to -42%; $P \leq 0.001$) and 24 h later (-3%). In the IF trial, β -CTX concentrations were significantly lower than baseline at 1 h post-exercise and remained below baseline until the end of the trial (-22 to -61%; $P \leq 0.01$). In the IF trial, β -CTX concentrations were increased above baseline 24 h later (+8%). In the DF trial, β -CTX concentrations were increased above baseline at 1 h post-exercise (+15%), then began to decrease and were significantly lower than baseline concentrations 3 and 4 h post-exercise (-44 to -65%; $P \leq 0.001$). In the DF trial, β -CTX concentrations were increased above baseline 24 h later (+8%) (Figure 19A).

At 1 and 2 h post-exercise, β -CTX concentrations were significantly lower in the IF trial than the DF ($P \leq 0.001$, $d=0.76$) and PLA trials ($P \leq 0.001$, $d=0.84$). At 3 h post-exercise, β -CTX concentrations were significantly higher in the PLA trial than the IF ($P \leq 0.001$, $d=1.13$) and DF trials ($P=0.026$, $d=0.54$). At 4 h post-exercise, β -CTX concentrations were significantly lower in the DF trial than the IF ($P=0.003$, $d=0.82$) and PLA trials ($P \leq 0.001$, $d=1.09$) (Figure 19A). The overall β -CTX response was significantly lower in the IF trial than the DF trial ($P=0.019$, $d=0.37$) and the PLA trial ($P \leq 0.001$, $d=0.84$).

7.3.5.2 P1NP

There was no main effect of *Trial* for P1NP, but there was a main effect of *Time* ($P \leq 0.001$) and there was a significant *Trial* \times *Time* interaction ($P \leq 0.001$). P1NP concentrations were significantly increased from baseline by the end of exercise in all trials (+32 to +33%; $P \leq 0.001$) and by 1 h post-exercise P1NP had decreased below baseline concentrations in all trials (-3 to -7%). In the PLA trial, P1NP concentrations remained below baseline until the end of the trial (-7 to -9%), but were increased above baseline 24 h later (+4%). In the IF trial, P1NP began to increase and reached concentrations above baseline at 3 and 4 h post-exercise (+1 to +3%) and 24 h later (+5%). In the DF trial, P1NP concentrations continued to decrease and by 3 and 4 h post-exercise were significantly lower than baseline (-10 to -11%; $P \leq 0.05$), but were increased above baseline 24 h later

(+4%) (Figure 19B). At 4 h post-exercise, P1NP was significantly higher in the IF trial than the DF ($P=0.026$, $d=0.20$) and PLA trials ($P=0.001$, $d=0.25$) (Figure 19B).

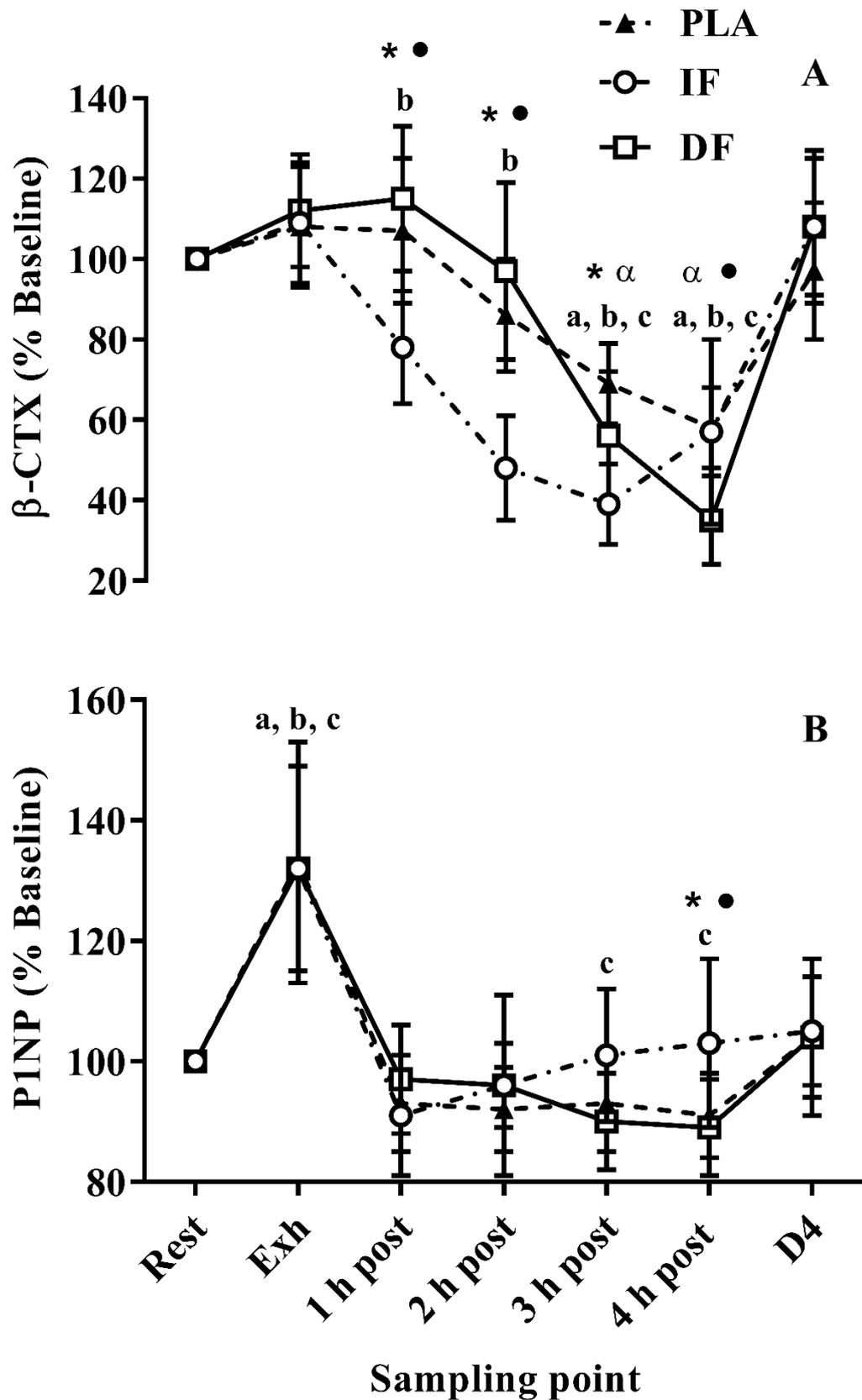


Figure 19. The percentage change in baseline concentrations of β -CTX (A) and P1NP (B), at Rest (Baseline), Exh (at exhaustion), 1 to 4 hours post-exercise and D4 (follow-up sample on D4), for PLA (filled triangles), IF (open circles) and DF (open squares). Data are mean \pm 1SD. ^a different ($P \leq 0.05$) from baseline (PLA) ^b different ($P \leq 0.05$) from baseline (IF), ^c different ($P \leq 0.05$) from baseline (DF). * IF different ($P \leq 0.05$) from PLA, ^a DF different ($P \leq 0.05$) from PLA, [•] IF different ($P \leq 0.05$) from DF.

Average bone turnover marker ratios were significantly higher in the IF trial (2.03 ± 0.98) compared to the DF trial (1.81 ± 1.01) ($P \leq 0.05$) and the PLA trial (1.39 ± 0.53) ($P \leq 0.001$) and were significantly higher in the DF trial compared to the PLA trial ($P \leq 0.001$).

7.3.6 Calcium metabolism

7.3.6.1 PTH

There was no main effect of *Trial* for PTH, but there was a main effect of *Time* ($P \leq 0.001$) and there was a significant *Trial* \times *Time* interaction ($P \leq 0.001$). PTH concentrations were significantly increased from baseline by the end of exercise in all trials (+124 to +131%; $P \leq 0.001$) but by 1 h post-exercise had decreased significantly below baseline concentrations in all trials (-17 to -37%; $P \leq 0.05$). In the PLA trial, PTH concentrations remained below baseline until the end of the trial (-3 to -15%) but were increased above baseline 24 h later (+4%). In the IF trial, PTH then began to increase and reached concentrations above baseline 3 and 4 h post-exercise (+2 to +7%) and 24 h later (+1%) (Figure 20A). In the DF trial, PTH continued to decrease and remained below baseline concentrations for the remainder of the trial (-13 to -27%) and 24 h later (-4%). At 3 h post-exercise, PTH was significantly higher in the IF trial than the DF trial ($P \leq 0.001$, $d=1.33$) (Figure 20A).

7.3.6.2 ACa

There was no main effect of *Trial* for ACa, but there was a main effect of *Time* ($P=0.003$) and there was a significant *Trial* \times *Time* interaction ($P=0.020$). ACa concentrations were increased from baseline by the end of exercise in all trials (+2 to +3%). In the PLA trial, ACa concentrations remained above baseline until the end of the trial (+2 to +4%) but had decreased below baseline 24

h later (-1%). In the IF trial, ACa remained above baseline (+2 to +3%) until 3 h post-exercise when ACa decreased below baseline (-3%), ACa then increased above baseline 4 h post-exercise (+1%) and remained there 24 h later. In the DF trial, ACa remained above baseline until the end of the trial (+2 to +4%) and returned to baseline 24 h later (Figure 20B). At 3 h post-exercise, ACa was significantly lower in the IF trial than the DF ($P=0.008$, $d=0.79$) and PLA trials ($P=0.001$, $d=0.98$) (Figure 20B).

7.3.6.3 Ca^{2+}

There was no main effect of *Trial* for Ca^{2+} , but there was a main effect of *Time* ($P\leq 0.001$) and there was a significant *Trial* \times *Time* interaction ($P\leq 0.001$). Ca^{2+} concentrations were significantly decreased below baseline by the end of exercise in all trials (-5 to -7%; $P\leq 0.001$). In the PLA trial, Ca^{2+} concentrations were still significantly below baseline by 1 h post-exercise (-4%; $P=0.002$) and remained below baseline until the end of the trial and 24 h later (-3%; $P=0.006$). In the IF trial, Ca^{2+} concentrations had returned to baseline by 1 h post-exercise (+1%) and remained at concentrations similar to baseline until the end of the trial and 24 h later (-1%). In the DF trial, Ca^{2+} concentrations had almost returned to baseline by 1 h post-exercise (-1%) and remained at concentrations similar to baseline until the end of the trial and 24 h later (-1%) (Figure 20C). At 1 h post-exercise, Ca^{2+} concentrations were significantly lower in the PLA trial than the IF trial ($P=0.010$, $d=1.41$) (Figure 20C).

7.3.6.4 PO_4

There was no main effect of *Trial* for PO_4 , but there was a main effect of *Time* ($P\leq 0.001$) and there was a significant *Trial* \times *Time* interaction ($P=0.007$). PO_4 concentrations were significantly increased above baseline by the end of exercise in all trials (+21 to +26%; $P\leq 0.001$). By 1 h post-exercise, PO_4 concentrations decreased below baseline in all trials (-5 to -13%). In the PLA trial, PO_4 concentrations continued to decrease at 2 h post-exercise (-8%), then increased and returned to baseline 3 h post-exercise. In the PLA trial, PO_4 concentrations were increased above baseline at 4 h post-exercise (+14%) and 24 h later (+3%). In the IF trial, PO_4 concentrations started to increase at 2 h post-exercise and increased above baseline 4 h post-exercise (+8%). In the IF trial, PO_4

concentrations were below baseline 24 h later (-2%). In the DF trial, PO₄ concentrations continued to decrease at 2 h post-exercise (-8%), concentrations started to increase thereafter, but remained below baseline until the end of the trial and 24 h later (-4%) (Figure 20D). At 1 h post-exercise, PO₄ concentrations were significantly lower in the IF trial than the DF trial ($P=0.049$, $d=1.03$) (Figure 20D).

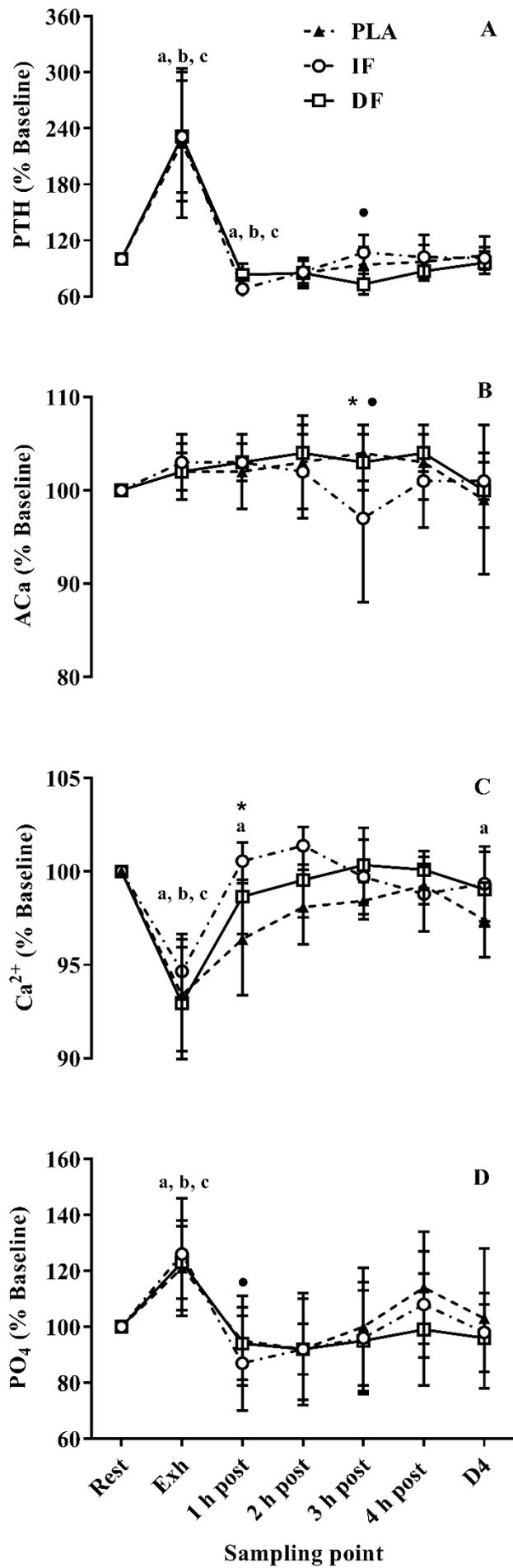


Figure 20. The percentage change in baseline concentrations of PTH (A), ACa (B), Ca^{2+} (C) and PO_4 (D) at Rest (Baseline), Exh (at exhaustion), 1 to 4 hours post-exercise and D4 (follow-up sample on D4), for PLA (filled triangles), IF (open circles) and DF (open squares). Data are mean \pm 1SD. ^a different ($P \leq 0.05$) from baseline (PLA) ^b different ($P \leq 0.05$) from baseline (IF), ^c different ($P \leq 0.05$) from baseline (DF). * IF different ($P \leq 0.05$) from PLA, ^a DF different ($P \leq 0.05$) from PLA, • IF different ($P \leq 0.05$) from DF.

7.3.6.5 Albumin

There was no main effect of *Trial* for albumin, but there was a main effect of *Time* ($P \leq 0.001$) and there was no *Trial x Time* interaction ($P = 0.054$). Overall mean albumin concentrations were significantly increased from baseline by the end of exercise (+3 to +4%; $P = 0.011$). There were no other significant changes in albumin concentrations (Figure 21).

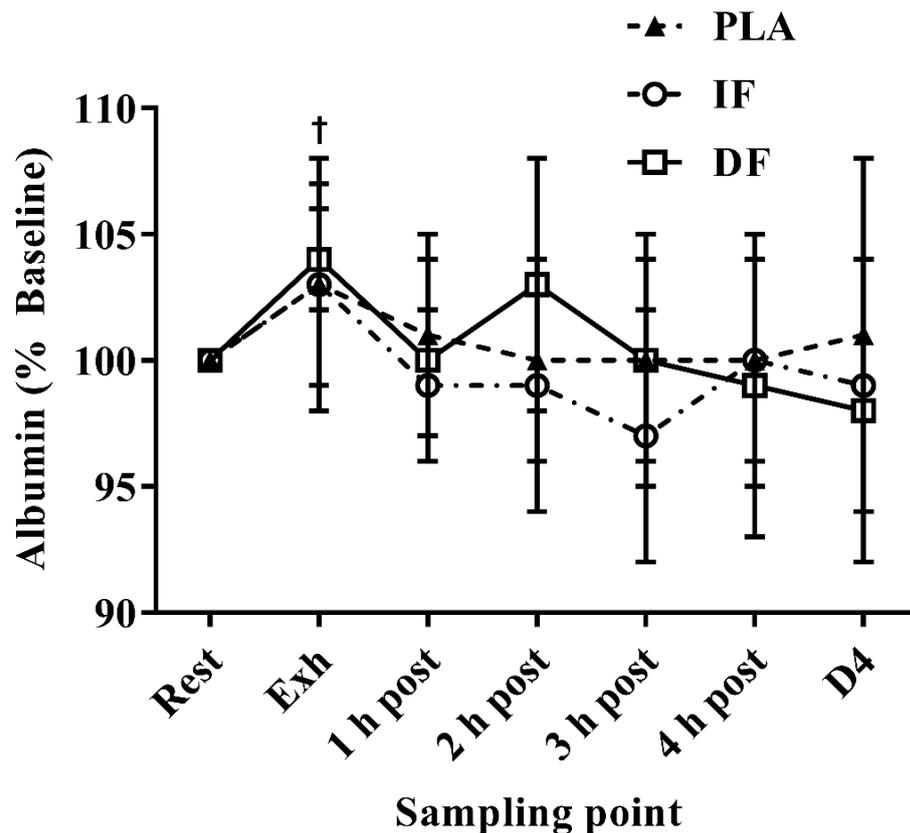


Figure 21. The percentage change in baseline concentrations of albumin at Rest (Baseline), Exh (at exhaustion), 1 to 4 hours post-exercise and D4 (follow-up sample on D4), for PLA (filled triangles), IF (open circles) and DF (open squares). Data are mean \pm 1SD. † overall mean concentrations different from baseline ($P \leq 0.05$).

7.4 Discussion

The main findings of the study are: 1) ingestion of the CHO+PRO solution containing 1.5 g·kgBM⁻¹ of CHO and 0.5 g·kgBM⁻¹ of PRO suppressed β -CTX concentrations following an exhaustive run, with a greater overall suppression when the CHO+PRO solution was ingested immediately; 2) immediate ingestion of the CHO+PRO solution resulted in small increases in P1NP concentrations at 3 and 4 h post-exercise; 3) delayed ingestion of the CHO+PRO solution (2 h post-exercise) also resulted in a large suppression of β -CTX concentrations. These findings are novel and have the potential to directly influence an athlete's dietary practices.

The response in the PLA trial, showed that the exhaustive running bout performed caused an immediate increase in bone turnover at the end of exercise, indicated by increased β -CTX and P1NP concentrations above baseline. This was followed by suppression of bone turnover during recovery, indicated by decreased β -CTX and P1NP concentrations below baseline. Ingestion of the CHO+PRO solution immediately post-exercise caused a rapid and prolonged (at least 4 h) suppression of β -CTX concentrations below baseline levels (-22 to -61%), whereas ingesting the PLA solution immediately post-exercise meant that β -CTX concentrations were increased above baseline by between +7 and +15%. When ingestion of CHO+PRO was delayed by 2 h, it caused suppression of β -CTX concentrations below baseline (-44 to -65%), which is similar to the suppression caused by immediate ingestion of the CHO+PRO solution and it occurred within the same timeframe, *i.e.*, 1 – 2 h after ingestion.

This rapid response is important because elite athletes habitually train multiple times a day, meaning that there are often only a few hours in between training sessions and therefore limited time for recovery and food consumption. Although the participants in the present study are not elite athletes, their trained nature means that the results are relevant and may be interpreted and used by elite athletes or support staff. The results indicate that post-exercise nutrient ingestion or exercise commencement can be timed so that the subsequent training session occurs when bone resorption is at its lowest and bone formation at its highest, *i.e.*, 3 – 4 hours after the first exercise bout with immediate ingestion of the CHO+PRO solution. This may maximise the anabolic and minimise the

catabolic bone response to the subsequent training session. Further research is needed to investigate whether this intervention does indeed produce a more anabolic environment for bone.

The significant increase in P1NP concentrations (+32 to +33%) at the end of exercise is interesting, as markers of bone formation are usually less responsive to acute bouts of exercise than markers of bone resorption (Guillemant *et al.*, 2004; Scott *et al.*, 2010; Scott *et al.*, 2012). Similarly, de Sousa *et al.* (2014) reported a 77% increase in P1NP concentrations after a high-intensity, interval running session (10 x 800m). In the present study, P1NP concentrations then decreased to below baseline levels at 1 h post-exercise in all trials, but the ingestion of the CHO+PRO solution immediately post-exercise caused P1NP to increase above baseline at 3 and 4 h post-exercise by between +1 to +3%. Whereas ingesting the PLA solution immediately post-exercise meant that P1NP remained below baseline concentrations by between -7 and -9%. When the CHO+PRO solution was ingested 2 h post-exercise, P1NP concentrations were suppressed further below baseline concentrations (-10 to -11%). It is possible that P1NP could have increased after the last measurement was taken but was missed by the sampling protocol, therefore it would be useful for future research to examine a longer post-exercise period to investigate the longer term response. The significantly increased P1NP concentrations at 4 h post-exercise in the IF trial compared to the DF and PLA trials is novel, and taken together, these results advocate the feeding of a CHO+PRO solution immediately post-exercise in order to reduce bone resorption and increase bone formation in the short-term recovery from intense exercise. Further, the average bone turnover marker ratios were above 1 in all three trials, indicating that bone turnover favoured bone formation. The significantly higher average bone turnover marker ratios in the IF trial compared to DF and PLA trials, and the significantly higher ratios in the DF compared to the PLA trial, suggests that both immediate and delayed consumption of the CHO+PRO recovery solution may be more beneficial for the bone turnover balance, compared to ingestion of a PLA solution. However, caution must be used when interpreting these bone turnover marker ratios, as they are simply a numerical interpretation of the bone turnover marker concentrations.

The results of the studies reported in Chapters 4 and 5 showed that the effects of certain training sessions may not persist to the following mornings, as there were no large changes in β -CTX or P1NP concentrations in any sample despite different training schedules on each day. Alternatively, the effects of all training sessions could have persisted to the following mornings, which may be the reason for the high concentrations of β -CTX and P1NP in all samples. In the present study, the effects of the CHO+PRO solution did not persist to the morning following exercise and β -CTX concentrations were increased in the IF and DF trials (+8%) compared to suppressed β -CTX concentrations in the PLA trial (-3%). P1NP was increased 24 h post-exercise in all trials (+4 to +5%). This increased bone turnover in the IF and DF trials may reflect the bones adapting to a possible hormonal response that is mediated by feeding. It is unlikely that the bones are adapting to the mechanical loading from the running bout alone, as β -CTX concentrations were not also increased 24 h post-exercise in the PLA trial. The hormonal mediators of this response are currently unknown. Clowes *et al.* (2002b) showed that short-term alterations in bone turnover may be due to the effects of hypoglycaemia, acute changes in PTH or regulatory hormones triggered by hypoglycaemia, however, this study was performed in resting individuals. Scott *et al.* (2012) and Sale *et al.* (2015) recently showed that GLP-2, leptin, ghrelin, insulin and cortisol are unlikely mediators of the effect of CHO or mixed meal feeding on bone turnover with exercise. Subsequently, this requires further research including the measurement of several other gastro-intestinal hormones. The regulation of the bone turnover response to feeding is complex, even at rest, and probably has several mediators (Walsh and Henriksen, 2010), which may be further complicated by an exercise bout.

Although this increased bone turnover response may be positive in sub-elite or trained athletes or recreationally active individuals, elite athletes that train multiple times a day with minimal recovery time and rest days are more likely to suffer from consistently increased bone turnover, which was shown in Chapters 4 and 5, and this may have detrimental effects on bone health and enhance the stress fracture risk (Parfit, 1982; Schaffler *et al.*, 1990; Riggs *et al.*, 1996; Oosthuysen *et al.*, 2013). The trained runners and triathletes that participated in the present study had mean resting β -CTX concentrations of $0.54 \pm 0.25 \text{ ng}\cdot\text{mL}^{-1}$ and P1NP concentrations of $65.23 \pm 30.81 \text{ ng}\cdot\text{mL}^{-1}$, which are

higher than the non-active, healthy population (de Papp *et al.*, 2007; Glover *et al.*, 2008; Glover *et al.*, 2009; Walkdron-Lynch *et al.*, 2010; Jenkins *et al.*, 2013; Michelsen *et al.*, 2013) (Appendix A). Furthermore, Chapters 4 and 5 showed that elite triathletes have mean resting bone turnover marker concentrations that are even higher than the trained runners and triathletes (Appendix A). This is supported by Oosthuysen *et al.* (2013) who showed that bone resorption and bone formation markers were significantly elevated each morning after four successive 3 h cycling bouts in trained cyclists. Although it is speculative at this stage, elite athletes may experience an imbalance between whole-body rates of resorption and formation or defective coupling (Parfit, 1982), meaning that neither bone resorption nor bone formation is performed adequately and the quality of the bone may be poorer, or they may experience accelerated remodelling, which can increase bone microdamage accumulation (Schaffler *et al.*, 1990; Mori and Burr, 1993; Burr, 2002) and stress fracture risk (Bennell *et al.*, 1996a; Riggs *et al.*, 1996; Burr *et al.*, 1997; Fredericson *et al.*, 2006). Indeed it should be noted that in a normal, healthy basic multicellular unit, the suppression of bone resorption may not always be desired, if the function of bone resorption is to breakdown and remove damaged bone at areas of microdamage accumulation to allow the area to be repaired and strengthened. Therefore, it is crucial for future research to investigate the long-term effects of post-exercise suppression of bone resorption on different athletic populations.

Ingestion of the CHO+PRO solution post-exercise is not sufficient to cause a prolonged suppression of bone resorption and/or elevation of bone formation. However, as elite athletes rarely go 24 h without a training session and often have a second session within four hours of finishing the first session, the bone turnover response 24 h post-exercise is less important than the immediate response as it does not reflect real life athlete practice. The more important time point is therefore, 4 h post-exercise, as this may be around the same time that the second training session would start. As we have now investigated the effect of post-exercise feeding after a single acute bout of exercise, future studies should investigate the effect of post-exercise feeding on repeated bouts of exercise occurring on the same day.

The responses of Ca^{2+} and PO_4 to exercise are in line with the results of the study reported in Chapter 6. The responses are only significantly different between trials at 1 h post-exercise; Ca^{2+} concentrations were lower in the PLA trial compared to the IF trial, suggesting that IF augments the recovery of Ca^{2+} to baseline concentrations, and PO_4 is lower in the IF trial compared to the DF trial. Transient peaks in PTH concentrations, as shown in the present study, are anabolic for bone (Tam *et al.*, 1982; Frolik *et al.*, 2003) and the study reported in Chapter 6 showed that PTH secretion during exercise and recovery is controlled by both Ca^{2+} and PO_4 , therefore these metabolites are likely to be mediating any anabolic effect of increased PTH concentrations. The fact that PTH and PINP follow the same response may suggest that PTH is mediating an anabolic response in the IF trial, although this response needs to be confirmed.

At 3 h post-exercise, PTH concentrations were greater in the IF trial than in the DF trial (+7% vs -27%). This response coincides with significantly lower ACa concentrations at 3 h post-exercise in the IF trial compared to the DF and PLA trials (-3% vs +3 to +4%). The action of increased PTH secretion is to increase calcium through mobilisation of the bone reservoir via activation of bone resorption (and also by increasing renal tubular reabsorption and intestinal calcium absorption) (McSheehy and Chambers, 1986; Brown, 2000; Zitterman *et al.*, 2002). β -CTX concentrations were at their lowest at 3 h post-exercise in the IF trial, suggesting that changes in PTH concentrations and calcium metabolism are unlikely to mediate the acute suppression of bone resorption by post-exercise CHO+PRO feeding.

The results of the study reported in Chapter 6 showed that ACa is unsuitable when investigating the rapid response of calcium metabolism to exercise, which may also be true when investigating CHO+PRO ingestion around exercise. Although Ca^{2+} (non-protein bound calcium) decreased at the end of exercise, because albumin concentrations increased, ACa was corrected accordingly and remained fairly unchanged throughout exercise, other than at 3 h post-exercise. Changes in albumin could have been affected by the ingestion of dietary protein throughout the recovery period, which has previously been shown to increase circulating albumin concentrations (Kaysen *et al.*, 1986; Kirsch *et al.*, 1995); however albumin did not change significantly throughout the recovery period

in the present study. The increase in albumin at the end of exercise could have been to encourage more calcium to be transported around the body, due to the tissues requiring additional Ca^{2+} to keep up with the demand in energy consumption, although the increase in albumin might also just reflect haemoconcentration as a result of the exercise to exhaustion. These data are uncorrected for plasma volume changes, which could influence the interpretation of the biological data obtained at the end of exercise and during the recovery period.

In conclusion, a practical recommendation for endurance athletes training multiple times a day could include the ingestion of a CHO+PRO recovery solution (containing around $1.5 \text{ g}\cdot\text{kgBM}^{-1}$ of high glycaemic index CHO and $0.5 \text{ g}\cdot\text{kgBM}^{-1}$ of whey PRO) immediately after a prolonged and intense running bout, to create a more positive bone turnover balance in the acute recovery period from exercise. However, we do not know whether long-term consumption of the CHO+PRO recovery solution and suppression of bone resorption will affect structural bone adaptation. Immediate ingestion of a CHO+PRO recovery solution is recommended because it will also allow more time for digestion before the start of a subsequent training session. The mechanisms underlying the acute changes in bone turnover remain unknown, but a change in calcium metabolism is unlikely to fully mediate the response. However it should be acknowledged that these results may only apply to conditions where participants are in a negative energy balance; given the total energy intake of $2,000 \text{ kcal}\cdot\text{d}^{-1}$ on day 3 combined with the exhaustive exercise bout, participants were likely to be in a negative energy balance. As Zanker and Swaine (2000) showed that bone turnover is only negatively affected with repeated running bouts when runners are in a negative energy balance, the effect of the CHO+PRO recovery solution could have had a more pronounced effect due to the negative energy balance that was likely to be present in this study.

CHAPTER 8: GENERAL DISCUSSION

The aims of this thesis were: 1) to identify what influences bone health in elite triathletes; 2) to identify possible areas for intervention to improve bone health and the subsequent risk of stress fracture injury; 3) to produce research that was applicable to elite athletes with the potential to directly influence athlete behaviour in relation to bone health. These aims were achieved by investigating bone turnover at two phases of the season in elite triathletes, along with, dietary intakes, energy expenditures and dermal calcium losses; by exploring the mechanism and timeframe of PTH and calcium regulation during exercise and recovery; and by investigating post-exercise nutrient ingestion on the bone turnover response.

It was important to provide an overview of the bone injury problem amongst the current British Triathlon team. As stress fractures can cause months of missed training and restricted weight bearing activity (Matheson *et al.*, 1987; Ranson *et al.*, 2010), there would be huge negative implications if a member of the Olympic team sustained a stress fracture in an Olympic year. The study reported in Chapter 4 showed that there have been 15 stress fracture injuries and 3 stress responses amongst 16 elite British triathletes. Twelve of the 16 triathletes had suffered at least 1 stress fracture and 3 of these 12 had suffered from 2 stress fractures throughout their athletic careers to date. The participants in the study reported in Chapter 4 represented 50% of the British Triathlon World Class Performance Squad. Considering the success of the British triathletes at the Rio Olympic Games – winning Gold, Silver and Bronze medals and a 4th place finish across the men's and women's races, it is reasonable to suggest that the group of triathletes that participated in the studies reported in this thesis truly represent the elite triathlete population and there are no other studies that have collected such data in a comparable population.

It is difficult to compare the stress fracture injury results to other studies, due to authors investigating stress fracture incidence over different time periods, using different methods to diagnose the injuries, differences in using prospective and retrospective methods and recruitment bias. Bennell *et al.* (1996b) used prospective methods and reported a high incidence of stress fractures across only 12 months in middle and long distance runners, particularly in females. The authors showed that 10 out

of 53 female athletes and 10 out of 58 male athletes sustained at least 1 stress fracture in the 12 month study, 50% of them occurred during the winter and the tibia and navicular were the most commonly injured bones. These results were similar to that of Zernicke *et al.* (1993) and Johnson *et al.* (1994) and who also used prospective methods. The retrospective evaluation used in Chapter 4 is a limitation of the study; however, the questionnaire results recorded by participants were in line with the injury data held by the sports science and medicine support staff at British Triathlon. This injury data also showed that there were 7 reports of medial tibial stress syndrome in the previous 18 months, and this overuse injury is often a precursor of a stress response (Detmer, 1986; Fredericson *et al.*, 1995; Anderson *et al.*, 1997; Beck, 1998; Galbraith *et al.*, 2009).

Cause and effect of stress fracture injury is almost impossible to establish, due to the multifaceted nature of bone remodelling and stress fracture development. The elite triathletes studied in this thesis suffered from a number of previously identified risk factors for stress fracture injury. As measured in Chapters 4 and 5, these primarily included; elevated bone turnover (Parfit, 1982; Schaffler *et al.*, 1990; Bennell *et al.*, 1996a; Bennell *et al.*, 1999; Burr, 2002; Schaffler, 2003), high training volumes (Rubin and Lanyon, 1984; Martin and McCulloch, 1987; Burr *et al.*, 2002; Bergman *et al.*, 2004; Robling, 2009), inadequate energy intakes that did not match the high energy expenditures, resulting in negative energy balances (Frusztajer *et al.*, 1990; Bennell *et al.*, 1995; Ihle and Loucks, 2004) and high dermal calcium losses (Klesges *et al.*, 1996; Barry and Khort, 2008; Barry *et al.*, 2011).

Bennell *et al.* (1999) proposed that accelerated bone remodelling, resulting from excessive bone strain or from the influence of systemic factors, may weaken bone because bone resorption occurs before new bone is formed, allowing for excessive accumulation of microdamage if repetitive mechanical loading is present at remodelling sites. The large volumes of training performed on a daily basis, the negligible amount of rest days taken and the large energy expenditures measured in 5 of the triathletes, means that repetitive mechanical loading is undoubtedly present in the triathletes. Average β -CTX concentrations in elite triathletes during off-season training (Chapter 4) were 2.4 times higher than the non-active population (de Papp *et al.*, 2007; Glover *et al.*, 2008; Glover *et al.*, 2009; Waldron-Lynch *et al.*, 2010; Jenkins *et al.*, 2013; Michelsen *et al.*, 2013) and 1.4 times higher

than recreationally active individuals (Musculoskeletal Physiology Research group; Appendix A). Average P1NP concentrations in elite triathletes were 2.1 times higher than the non-active population and 1.4 times higher than recreationally active individuals. Average PTH and ACa concentrations were lower in elite triathletes compared to recreationally active individuals and PO₄ concentrations were similar. Average β-CTX concentrations in elite triathletes during pre-competition training (Chapter 5) were 2.1 times higher than the non-active population and 1.3 times higher than recreationally active individuals. Average P1NP concentrations in elite triathletes were 1.7 times higher than the non-active population and 1.2 times higher than recreationally active individuals. Average PTH, ACa and PO₄ concentrations were lower in elite triathletes compared to recreationally active individuals. In addition, the bone turnover marker ratios were higher during pre-competition training compared to off-season training, indicating a shift in the bone turnover response favouring bone formation. The higher bone turnover shown during phases of increased training load is consistent with the results of other studies, although to our knowledge there are only two other studies that have investigated this in elite athletes (Jürimäe *et al.*, 2006; Lombardi *et al.*, 2011) so there is limited data available for comparison.

A limitation of the studies reported in Chapters 4 and 5 was the lack of a resting baseline sample, which was not possible to obtain from the triathletes as they did not have any rest days incorporated into their training schedules and them abstaining from a training day was not possible at any point during the season due to the importance placed on high training volumes during an Olympic year. This meant that interpretation of the bone turnover marker concentrations and other metabolite concentrations was difficult. Further, there are no reference ranges for other elite athletes; hence we compared the concentrations to resting ranges from other populations. However, these comparisons should be interpreted with caution, as the data from the recreationally active individuals are from resting blood samples, where the individuals refrained from exercise for a minimum of 24 hours prior to the blood sample, whereas, the triathletes' blood samples were taken after consecutive days of exercise. With this in mind, using samples taken the day after an exercise bout may have been more appropriate to use as a comparison, however, using this as the comparative sample would induce more variability. The different exercise modes, durations and intensities performed would

add multiple sources of variation compared to a resting sample, further, it would be difficult to match control participants to the extreme mechanical loading patterns performed by the triathletes. Despite this limitation there were numerous strengths of the studies reported in Chapters 4 and 5; primarily, the strict control of blood sampling and other measurements whilst in a real-life training environment and the elite nature of the participants, producing both reliable and ecologically valid results. Future studies should develop reference ranges for bone turnover marker concentrations amongst different elite athletes and should also develop a standard study protocol for the investigation of bone turnover throughout an athletic season, which will allow improved comparison of bone turnover between different athletes and sports.

Bennell *et al.* (1998) evaluated baseline bone turnover markers (OC, u-Pyr and u-NTX) in 95 track and field athletes and showed that the 20 athletes that developed a stress fracture had similar baseline and monthly concentrations to those that did not sustain a stress fracture. These results suggest that single monthly measurements of bone turnover markers are not useful predictors of stress fracture injury. However, this does not mean that bone turnover markers cannot reflect whole body changes in bone turnover, with local bone turnover likely to be occurring at stress fracture sites or the sites that experience the most mechanical loading and therefore microdamage (Bennell *et al.*, 1999). Assuming blood samples are taken under identically controlled conditions, monthly samples could show when an individual has accelerated or suppressed bone turnover, both of which could lead to the development of a stress fracture in individuals with high training loads (Martin, 1992; Bennell *et al.*, 1998; Schaffler, 2003).

However, simply knowing whether bone turnover is accelerated or suppressed will not predict if and when a stress fracture injury will develop, as there have been no threshold studies performed in humans *in vivo* (Mashiba *et al.*, 2000; Mashiba *et al.*, 2001; Schaffler, 2003). Therefore, the sampling protocol used in the studies reported in Chapters 4 and 5 would not predict if or when a stress fracture injury would occur, it could however identify at what phase of the season an athlete has an increased risk of bone injury. It may also allow certain athletes with greater bone injury risk to be identified, such as participant 13 in the studies reported in Chapters 4 and 5. This information is important for

coaches and sports science and medicine support staff as they will be able to alter an athlete's training load or external environment at particular times to reduce the risk of a bone injury. Consequent to identifying the high bone turnover marker concentrations in participant 13, this triathlete began working closely with a performance nutritionist, to improve dietary practices that may help reduce the risk of bone injury. Considering that these injuries can cause weeks or months of missed training, preventing them will help maximise available training time and is of utmost importance during an Olympic year.

Energy intakes were suboptimal compared to the energy expended, and CHO and PRO intakes were suboptimal compared to intakes reported in other studies of triathletes (Burke and Read, 1987; Nogueira and Da Costa, 2004) and compared to recommended guidelines for endurance athletes (Burke *et al.*, 2011). It is vitally important that athletes match energy intakes with energy expenditures as closely as possible, as the bone formation and bone resorption processes are negatively affected even with slight deficits (Ihle and Loucks, 2004). As a result of the study reported in Chapter 4, 5 of the triathletes now have specific target intakes to reach during off-season training, which is closely monitored by a performance nutritionist and allows the triathletes to accurately match energy intakes with energy expenditures. Energy expenditures were not measured in all 16 athletes, which is a limitation of the study. This means that the other 11 triathletes do not have individual target intakes based on energy expenditures, however, as training loads were similar between all athletes these data still provide useful information and a performance nutritionist can use the dietary information for educational purposes. Future studies should measure energy expenditures at different phases of the season, to provide specific target intakes for athletes throughout a whole season, which will allow the different training schedules to be adequately supported.

Due to the suspicion that underreporting of diet logs was present in Chapter 4, the 8 triathletes that also participated in Chapter 5 were briefed on the issues of underreporting and were encouraged to record dietary intakes in more detail during the pre-competition testing period. In hindsight, this was a limitation of the study and made the comparison of diet logs between testing periods less reliable, however, it is not certain that the same extent of underreporting would have occurred in each testing

period regardless. The use of the diet logs for educational purposes would not have been possible if there was drastic underreporting in both testing periods, which was an important consideration given that one of the aims of the thesis was to produce research that directly influences athlete behaviour. This was an instance where the Fast Practitioner and Slow Researcher Model (Section 1.1) became apparent; it was more important to provide applicable information that could be directly used by athletes and coaches, than to produce a reliable comparison that would not be beneficial or useful for athletes and coaches. Another issue and limitation that arose from athlete or coach reporting was the lack of detail in training logs. There was a lack of training intensity reported; athletes and coaches only tend to record training duration on a daily basis for simplicity and to ensure adherence. Future studies should explore the use of more objective methods to record training volume and intensity in triathlon, such as the use of power meters, global positioning systems or heart rate monitors.

The studies reported in Chapters 4 and 5 showed that the greatest total dermal calcium loss was 214 mg during a long cycle and the greatest rate of loss was 248 mg·h⁻¹ during a hard run, during the pre-competition training phase (participant 08). Sweat rate and composition varies by body region (Sripanyakorn *et al.*, 2009) and there are no studies that have compared dermal calcium losses via patch technique and whole body wash down. Further, the sweat analysis technique used in this thesis has not yet been validated, which will require a large population study that was outside the scope of this thesis. This means that the results were only gross estimates of dermal calcium losses, which is a limitation of the studies reported in Chapters 4 and 5. Therefore, future studies should validate and confirm a method for measuring dermal calcium losses in athletes. These large estimated losses are important because it has been shown that deviations from calcium homeostasis cause increases in PTH, which may subsequently increase bone resorption (Brown, 2000; Guillemant *et al.*, 2004; Barry and Khort, 2007). Sherk *et al.* (2013) showed that dermal calcium losses and decreases in serum Ca²⁺ were greater when a calcium supplement (1000 mg) was consumed after a cycling time trial, compared to consuming it before. Consuming the calcium supplement before cycling attenuated the PTH response but had no effect on the bone resorption response. The calcium supplement may have provided greater calcium availability in the gut during the cycling time trial, attenuating the decrease in serum Ca²⁺ concentrations. Oosthuysen *et al.* (2013) showed that when sweat calcium

excretion was around $50 \text{ mg}\cdot\text{h}^{-1}$, this resulted in a significant decrease in Ca^{2+} concentrations from pre- to post-exercise. Although, when sweat calcium excretion was below $40 \text{ mg}\cdot\text{h}^{-1}$, Ca^{2+} concentrations did not significantly decrease, suggesting that there may be a threshold or dose response relationship between dermal calcium losses and serum Ca^{2+} concentrations, which needs to be investigated. Future studies that prove that dermal calcium losses cause deviations from calcium homeostasis are required and further research is needed to determine whether the bone resorption response is related to dermal calcium losses and decreased serum Ca^{2+} during and after exercise, if the decrease is not attenuated by increased calcium availability in the gut.

The results of Chapter 4 showed that PTH increased in some participants at site 1, the morning after the longest and hardest training day of the week, compared to PTH concentrations on other days. This training day involved a long cycle and a hard run, which were the training sessions that caused the greatest total calcium loss and highest rate of calcium loss, respectively. Similarly, the results of Chapter 5 showed that PTH increased in some participants the morning after an evening track session, compared to PTH concentrations on other days. Pre-exercise calcium ingestion has been shown to attenuate increases in bone resorption (Guillemant *et al.*, 2004) and PTH (Barry *et al.*, 2011; Sherk *et al.*, 2013). Although Guillemant *et al.* (2004) and Barry *et al.* (2011) showed that consuming 2 L of high calcium water before and during exercise was an effective strategy to minimise the activation of the regulatory mechanisms, this is a challenging and unrealistic practice for triathletes to adopt on a daily basis. Triathletes would rarely consume such large volumes of fluid during training due to gastrointestinal issues and limited opportunity. Given the efficacy of pre-exercise calcium supplementation shown in previous studies, individualised calcium supplementation may be an effective strategy to implement around long cycles and hard runs in triathletes. However, to be effective and practical this must be in the form of a calcium supplement or dairy foods. Haakonssen *et al.* (2014) showed that a calcium-rich (1,350 mg) breakfast consumed 90 minutes before a cycling bout attenuated the increase in PTH and β -CTX, however there are no studies that have investigated calcium ingestion prior to running sessions, where gastrointestinal discomfort may be a more important issue.

It was important to understand the mechanism that controls PTH and calcium regulation during exercise and recovery before any individualised calcium supplementation protocols can be implemented in elite athletes. The study reported in Chapter 6 was the first study to explore this mechanism in detail during running. The Ca^{2+} response shown in Chapter 6 supports the findings of other studies (Maïmoun *et al.* 2005; Maïmoun *et al.* 2009; Barry *et al.* 2011); intense exercise causes a decline in serum Ca^{2+} concentrations at the end of exercise. Dermal, urinary and gastrointestinal calcium losses may have exceeded intestinal calcium absorption during exercise (Barry *et al.*, 2011) which coincided with increased PTH concentrations, to restore serum calcium levels. Future studies should now examine individualised pre-exercise calcium supplementation in elite triathletes to investigate if this prevents decreased serum Ca^{2+} concentrations and subsequent increases in bone resorption during and after an intense running session.

The results of Chapter 6 showed that changes in Ca^{2+} and PO_4 occur in close temporal relation to changes in PTH and are likely to control PTH secretion during exercise and recovery. This was the first study to show that PTH transiently decreases at the onset of exercise, then increases throughout exercise and peaks in the first minutes of recovery. These findings are important because identifying the peak in PTH concentrations shortly after the cessation of a running bout will allow specific training sessions to be developed. These could involve high intensity interval running bouts that will cause acute increases in PTH concentrations, followed by rest periods that will allow PTH concentrations to return to baseline. This may promote the anabolic actions of short-lived elevated PTH concentrations (Dempster *et al.*, 1993; Dempster *et al.*, 2001). To elucidate, peak PTH concentrations were shown to occur 5 minutes after 30 minutes of running at 75% $\text{VO}_{2\text{max}}$, with PTH concentrations then decreasing rapidly after reaching peak concentrations, so a second running bout could commence when PTH concentrations are declining. This could be combined with an endurance athlete's long distance, low intensity runs. However, consideration should be given to an athlete's total training volume or schedule, as if these high intensity running sessions were an addition to a training programme they would increase total energy expenditure and may decrease energy availability. Therefore, the use of such training sessions should be considered for each athlete, against their existing training schedule and their risk of bone injury.

Studies investigating the effect of repeated running bouts on the PTH response are necessary before detailed high intensity interval training sessions, focused on bone adaptation, can be planned and applied by coaches and athletes, as this has not yet been thoroughly investigated. Bouassida *et al.* (2003) and Scott *et al.* (2011) explored the effect of two running bouts, but there were a number of limitations with the study by Bouassida *et al.* (2003). Such as, a lack of dietary control and a questionable sampling protocol; blood samples were taken at different points in the two trials and at different times of the day, making it difficult to compare the single and double running bouts. The effect of multiple exercise bouts, with varying exercise and recovery durations should now be investigated.

The effect of PTH on long-term bone health in athletes is unclear due to the dual action of PTH, which seems to be dependent on the duration that PTH concentrations are elevated for (Frolik *et al.*, 2003). Two possible PTH related interventions that could have positive influences on bone, were highlighted in earlier paragraphs. One involved suppressing the PTH response to exercise using pre-exercise calcium supplementation, and the other involved modulating the PTH response to exercise using repeated high intensity running bouts. Pre-exercise calcium supplementation may be useful prior to long training sessions when a prolonged increase in PTH may become catabolic for bone (Tam *et al.*, 1982; Tsai *et al.*, 1984; Ljunghall *et al.*, 1986; Ljunghall *et al.*, 1988; Hock and Gera 1992; Grimston *et al.*, 1993; Uzawa *et al.*, 1995; Chappard *et al.*, 2001). However, the studies reported in Chapters 4 and 5 showed that hard running sessions, that would be the sessions that most closely reflects a high intensity interval running session, caused the greatest rates of dermal calcium losses, potentially causing the greatest perturbation in calcium homeostasis and therefore the greatest increases in PTH and bone resorption (Brown, 2000; Brown, 2013). The problem with these potential interventions is that the resultant long-term effect on the bones is unknown. It is not known whether suppression of the PTH response to hard running sessions would be beneficial, or if the natural PTH response would have anabolic effects. This highlights the need to investigate the long-term effects of different types of training sessions on bone health and structural adaptation; however this would be difficult to do, particularly in elite athletes.

There are also a number of other mechanistic studies that are required before PTH regulation during exercise and recovery is completely understood. These include investigating the initial decrease in PTH from baseline to 5 minutes of exercise, as the study in Chapter 6 was the first study to observe this initial response. The initial increase in Ca^{2+} at the two higher exercise intensities but a lack of an increase in the lower exercise intensity was also a novel finding that requires further exploration. The difference in the PTH response to cycling and running should also be investigated, as this may vary due to different mechanical loading (non-weight-bearing vs weight-bearing exercise).

The training volumes in the studies reported in Chapters 4 and 5 showed that the triathletes had congested training schedules and this meant that they would have had limited time to consume meals throughout a training day. This meant that they would often consume the majority of their energy in the late evenings after their last training session of the day. Because of the huge energy expenditures recorded, it would have taken a considerable amount of time to shop for, prepare, cook, consume and digest that amount of energy in normal food, which the training schedule did not allow for. This meant that the triathletes were often likely to perform training sessions in under-fuelled and under-recovered states. An area for intervention that was identified in the studies reported in Chapters 4 and 5 was the post-exercise period, which was also identified based on the results of previous studies investigating other exercise related nutritional interventions (Scott *et al.*, 2012; Sale *et al.*, 2015). Consuming energy dense nutrients, containing little fibre and fat in the immediate post-exercise period would leave enough time for digestion prior to a subsequent training session (Rehrer *et al.*, 1992; Jenkins *et al.*, 2009) and would also allow other recovery goals to be met, such as glycogen resynthesis and protein synthesis (Jentjens and Jeukendrup, 2003; Tipton *et al.*, 2004).

The study reported in Chapter 7 showed that the ingestion of a CHO+PRO recovery solution immediately after a prolonged and intense running bout caused a rapid suppression of β -CTX and small increases in PINP. To our knowledge this was the first study that has investigated post-exercise CHO+PRO ingestion on the acute bone turnover response, and the small increase in PINP with immediate ingestion was a novel finding that has not been shown with pre-exercise and during

exercise nutrient ingestion (Scott *et al.*, 2012; Sale *et al.*, 2015). The increase in P1NP concentrations is encouraging as it means that the bone turnover balance is shifting towards increased bone formation, rather than simply suppressing bone resorption. This is a simple and practical intervention that can be easily implemented by athletes and coaches after training sessions. A limitation of the study reported in Chapter 7 was the lack of measurement of gastrointestinal hormones, which were not measured due to cost implications. This means that the results of Chapter 7 do not contribute to the understanding of the mechanisms underlying the acute changes in bone turnover with exercise and nutrient ingestion. There was also no subjective measurement of gastrointestinal discomfort in Chapter 7, which would have been useful to assess the tolerability of the post-exercise CHO+PRO recovery solution. Additionally, there was no measurement of plasma volume shifts, in this study or any other study in this thesis, meaning that concentrations of blood parameters may have been altered by haemoconcentration or haemodilution, however the concentrations presented were what the tissue was exposed to.

It is important for future studies to investigate the effect of post-exercise nutrient ingestion with multiple exercise bouts, as this more closely reflects training schedules of elite athletes. It is also important that future studies investigate the long-term effects of post-exercise suppression of bone resorption. The function of bone resorption is to breakdown and remove damaged bone at areas of microdamage accumulation to allow the area to be repaired and strengthened, therefore suppression of bone resorption may not be desired in all instances. This is the same issue as suppressing the PTH response to exercise, and it will be unknown whether suppression of the natural bone turnover response to exercise is beneficial in athletes until the long-term effects of different types of training sessions have been explored.

8.1 Research impact on British Triathlon

It is important to highlight the impact that this programme of research has had on the British triathletes, as one of the aims of this thesis was to produce research that is applicable to elite athletes and has the potential to directly influence athlete behaviour. After feedback of the results of this

thesis to the 16 triathletes that participated in the studies reported in Chapters 4 and 5, British Triathlon coaches and sports science and medicine support staff, all members have become more aware of the issues of poor bone health and stress fracture injury amongst the triathletes. The research has led to a bone health working group being established, where coaches and support staff meet to discuss issues relating to bone health, but also related to the female athlete triad and RED-S and how the current situation can be managed and improved.

To date, 3 triathletes have started to supplement with calcium prior to long cycling sessions and hard running sessions. Total calcium intakes and the timing of ingestion in relation to training sessions has become a focus for the performance nutritionist, which therefore requires further research to individualise, validate and refine this. The energy expenditure data has been used to set individual target intakes during off-season training for the 5 triathletes that used DLW, and the energy intake data has been used to educate all triathletes and create subsequent nutrition plans based on this dietary information. All triathletes that were at the pre-Rio Olympics altitude camp were provided with immediate CHO+PRO recovery drinks after hard running sessions, all triathletes are also encouraged to do this on a regular basis during normal training. The recovery drinks have been well tolerated and do not cause any gastrointestinal discomfort in subsequent training sessions. The impact that this research has had on the members of the British Triathlon team was recently expressed by a coach; “This research has influenced their (the Loughborough based triathletes) behaviour more than any other nutritional intervention we have ever tried”.

Table 24. Table showing how this thesis has contributed and added to the research area of bone health in athletes.

Bone health in athletes research prior to this thesis:	This thesis has added the following evidence:
Limited bone turnover measurements in athletes at different phases of the season (rowers and skiers only)	Multiple bone turnover measurements in elite triathletes at different phases of the season (Chapters 4 and 5)
Poorly controlled field studies around acute bouts of exercise (often marathons or ultramarathons)	Controlled measures taken in a real-life athlete training environment (Chapters 4 and 5)
Stress fracture injury prevalence data in some groups of athletes (mainly track and field athletes and middle and long distance runners)	Stress fracture injury data in elite triathletes (Chapter 4)
RED-S identified but limited research to show that RED-S occurs in male athletes	Evidence of RED-S in elite male triathletes (high bone turnover and negative energy balances) (Chapters 4 and 5)
Limited dermal calcium loss data in athletes (basketball players and laboratory cycling time trials only)	Dermal calcium loss data in elite triathletes during a range of training sessions in a real-life athlete training environment (Chapters 4 and 5)
Studies confirming the mechanism of PTH regulation at rest only	Evidence of the mechanism controlling PTH regulation during exercise and recovery (Chapter 6)
Studies examining pre-exercise and during exercise feeding on the bone turnover response	Evidence of the effect of post-exercise feeding on the bone turnover response (Chapter 7)
BMD measurements in some groups of athletes (mostly acute measures)	

8.2 Conclusions

The studies reported in Chapters 4 and 5 showed that elite British triathletes have accelerated bone turnover during both off-season and pre-competition training, compared to recreationally active individuals and non-active, healthy individuals. Although cause and effect cannot be established from the current programme of work, high energy expenditures, insufficient energy intakes and high rates of dermal calcium loss, may contribute to this accelerated bone turnover. Bone turnover was lower during pre-competition training compared to off-season training, which may be due to increased total energy, calcium, CHO and PRO intakes in pre-competition training compared to off-season training. Despite greater dermal calcium losses in pre-competition training, the higher energy intakes probably superseded this effect on bone turnover. The rate of dermal calcium loss was highest during running sessions in both phases of the season, which suggests that the timing of calcium ingestion around these training sessions may be more important than overall calcium intakes across a day (as long as overall intakes are sufficient to prevent a negative calcium balance). The participants in these studies compete at World Triathlon Series level or above and represented 50% of the British Triathlon World Class Performance squad, therefore the results apply directly to elite triathletes.

The study reported in Chapter 6 was the first study to investigate the temporal pattern of PTH regulation during exercise and recovery. The results showed that, at the onset of exercise, PTH transiently decreased and then increased throughout exercise, peaking in the first minutes of recovery, before decreasing below baseline concentrations during ongoing recovery. Changes in Ca^{2+} and PO_4 occurred in close temporal relationship to changes in PTH. Cross-correlational analysis showed that PTH secretion during exercise and recovery is controlled by a combination of changes in Ca^{2+} and PO_4 and that the mechanism might be different during exercise and recovery. These data can be used to inform further investigations that explore dermal calcium losses and the response to calcium supplementation before or during training sessions in athletes.

The study reported in Chapter 7 showed that a CHO+PRO recovery drink consumed immediately post-exercise is beneficial for endurance athletes, to facilitate a more positive bone turnover balance

in the acute recovery period, by suppressing bone resorption and increasing bone formation. This nutritional intervention was subsequently employed by the British Triathlon squad and has become a routine post-exercise practice.

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APPENDICES

Appendix A Resting bone turnover marker concentrations

Average resting concentrations for non-active, healthy premenopausal women and non-active, healthy men.

	β -CTX (ng·mL ⁻¹)	P1NP (ng·mL ⁻¹)
de Papp <i>et al.</i> (2007) Women	0.28	39.50
Glover <i>et al.</i> (2008) Women	0.30	35.90
Glover <i>et al.</i> (2009) Women	0.32	38.70
Waldron-Lynch <i>et al.</i> (2010) Men	N/A	49.40
Jenkins <i>et al.</i> (2013) Men	0.36	41.00
Jenkins <i>et al.</i> (2013) Women	0.26	31.00
Michelsen <i>et al.</i> (2013) Men	0.28	38.00
Michelsen <i>et al.</i> (2013) Women	0.23	36.70

Resting concentrations for recreationally active individuals collected by the Musculoskeletal Physiology Research group at Nottingham Trent University (Results from the study reported in Chapter 6; Sale *et al.*, 2015; Papageorgiou *et al.*, 2016; Unpublished thesis studies entitled; Genetic Associations with Bone Turnover Following 120 Minutes of Treadmill Running; The Effect of Reduced Energy Availability Through Diet and Exercise on Bone Turnover Markers).

	β -CTX (ng·mL ⁻¹)	P1NP (ng·mL ⁻¹)	PTH (pmol·L ⁻¹)	ACa (mmol·L ⁻¹)	PO ₄ (mmol·L ⁻¹)
Average	0.51	61.40	3.23	2.41	1.21
SD	0.22	25.89	1.14	0.26	0.17
Minimum	0.13	24.95	1.00	2.15	0.75
Maximum	1.34	164.82	8.29	3.17	1.71

Resting concentrations for elite triathletes (results from studies reported in Chapters 4 and 5).

	β -CTX (ng·mL ⁻¹)	P1NP (ng·mL ⁻¹)	PTH (pmol·L ⁻¹)	ACa (mmol·L ⁻¹)	PO ₄ (mmol·L ⁻¹)
Average	0.69	81.14	3.03	2.33	1.18
SD	0.29	33.31	0.65	0.08	0.18
Minimum	0.34	42.45	1.77	2.18	0.82
Maximum	1.76	178.00	5.43	2.49	1.66

Appendix B Health screening questionnaire

Name or Number

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 <input type="checkbox"/> | No <input type="checkbox"/> |
| (b) attending your general practitioner | Yes <input type="checkbox"/> | No <input type="checkbox"/> |
| (c) on a hospital waiting list | Yes <input type="checkbox"/> | No <input type="checkbox"/> |

2. **In the past two years**, have you had any illness which require you to:

- | | | |
|---|------------------------------|-----------------------------|
| (a) consult your GP | Yes <input type="checkbox"/> | No <input type="checkbox"/> |
| (b) attend a hospital outpatient department | Yes <input type="checkbox"/> | No <input type="checkbox"/> |
| (c) be admitted to hospital | Yes <input type="checkbox"/> | No <input type="checkbox"/> |

3. **Have you ever** had any of the following?

- | | | |
|--------------------------|------------------------------|-----------------------------|
| (a) Convulsions/epilepsy | Yes <input type="checkbox"/> | No <input type="checkbox"/> |
| (b) Asthma | Yes <input type="checkbox"/> | No <input type="checkbox"/> |
| (c) Eczema | Yes <input type="checkbox"/> | No <input type="checkbox"/> |
| (d) Diabetes | Yes <input type="checkbox"/> | No <input type="checkbox"/> |
| (e) A blood disorder | Yes <input type="checkbox"/> | No <input type="checkbox"/> |
| (f) Head injury | Yes <input type="checkbox"/> | No <input type="checkbox"/> |
| (g) Digestive problems | Yes <input type="checkbox"/> | No <input type="checkbox"/> |
| (h) Heart problems | Yes <input type="checkbox"/> | No <input type="checkbox"/> |

- (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

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 (e.g. to confirm problem was/is short-lived, insignificant or well controlled.)

.....

.....

.....

ARTD LIFESTYLE QUESTIONNAIRE

Your answers to this questionnaire are confidential and will not be seen by anyone other than the study team. The information is to be used **only** for the study, and **is not part** of your course or assessment. Please be as honest and accurate as you can with your answers. Please place a cross or a number (depending on the question) in the box next to your chosen answer and do not leave any questions blank.

How do I fill in the questionnaire?

To indicate your response place a **CROSS** in the box e.g. X

To cancel a response fill in the box e.g. and record the new response with a cross.

If you have any questions please ask.

Thank you.

Personal Details

Q1 Today's Date:

D	D	/	M	M	/	Y	Y	Y	Y
---	---	---	---	---	---	---	---	---	---

Q2 Subject Number:

--	--	--	--	--	--	--	--	--	--

Q3 Group:

--

Q4 Which of the following most closely describes your ethnic background?

- Any White background
- Any Black background
- Any Asian background
- Any Chinese background
- Any Mixed background
- Any Other background

Q5 Date of Birth:

D	D	/	M	M	/	Y	Y	Y	Y
---	---	---	---	---	---	---	---	---	---

Q6 Sex

- Male
- Female

If Other please state:

--

For official use only:

Height: _____

Weight: _____



Medical History

Indicate your response with a cross in the box e.g.

Q15 Have you ever been told by a doctor that:

- | | Yes | No | Not Sure |
|---|--------------------------|--------------------------|--------------------------|
| a) You are underweight | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| b) You have suffered from an eating disorder, e.g. anorexia, bulimia | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| c) You suffer from anaemia (i.e. have been advised to take iron supplements) | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| d) You have broken or fractured a bone/bones | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| e) You have suffered a stress fracture
(an injury to a bone caused from over exercising) | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |

Q16 If you have broken or fractured a bone/bones:

- a) How many times have you broken a bone?
- b) Which bones have you broken? (e.g. a bone in your upper arm or lower leg)
- c) How old were you when you last broke a bone?
- d) Was it confirmed by an x-ray?
- Yes
 No
 Not Sure

Q17 If you have been told by a doctor that you have suffered a stress fracture: *

- a) How old were you when you last suffered a stress fracture?
- b) Which side(s) was the stress fracture?
 L R
- c) List which bones:
- d) Was it confirmed by a bone scan?
- Yes
 No
 Not Sure

Q18 (a) Where did you live for most of your life UP TO THE AGE OF 13?

Country

City/Town

Q18 (b) How long did you live there for?

years

Q19 (a) Where did you live for most of your life FROM THE AGE OF 13?

Country

City/Town

Q19 (b) How long did you live there for?

years



Sunlight Exposure

Indicate your response with a cross in the box e.g.

Q20 When you spend time in the sun without sun cream, do you:

- a) Always burn, never tan
- b) Usually burn, tan with difficulty
- c) Sometimes burn, but gradually tan
- d) Rarely burn, tan easily
- e) Never burn, always tan

Q21 When you spend time in the sun, how often do you wear sun cream?

- | | | |
|-----------------------------------|--------------------------|--------------------------|
| | On holiday | Any other time |
| a) Never | <input type="checkbox"/> | <input type="checkbox"/> |
| b) Sometimes | <input type="checkbox"/> | <input type="checkbox"/> |
| c) Most times | <input type="checkbox"/> | <input type="checkbox"/> |
| d) All the time | <input type="checkbox"/> | <input type="checkbox"/> |
| e) I do not spend time in the sun | <input type="checkbox"/> | <input type="checkbox"/> |

Q22 What level of sun protection (spf) do you generally use?

If not listed, cross the closest one

- | | | |
|---------------|--------------------------|--------------------------|
| | On holiday | Any other time |
| a) 15 or less | <input type="checkbox"/> | <input type="checkbox"/> |
| b) 20 or 25 | <input type="checkbox"/> | <input type="checkbox"/> |
| c) 30 or 35 | <input type="checkbox"/> | <input type="checkbox"/> |
| d) 40 | <input type="checkbox"/> | <input type="checkbox"/> |
| e) 50 or more | <input type="checkbox"/> | <input type="checkbox"/> |
| e) Unsure | <input type="checkbox"/> | <input type="checkbox"/> |
| f) None | <input type="checkbox"/> | <input type="checkbox"/> |

Q23 Where do you usually apply sun cream? (Cross all the boxes that apply)

- | | | |
|--|--------------------------|--------------------------|
| | On holiday | Any other time |
| a) All over | <input type="checkbox"/> | <input type="checkbox"/> |
| b) Face and neck | <input type="checkbox"/> | <input type="checkbox"/> |
| c) Shoulders | <input type="checkbox"/> | <input type="checkbox"/> |
| d) Arms | <input type="checkbox"/> | <input type="checkbox"/> |
| e) Chest | <input type="checkbox"/> | <input type="checkbox"/> |
| f) Back | <input type="checkbox"/> | <input type="checkbox"/> |
| g) Stomach | <input type="checkbox"/> | <input type="checkbox"/> |
| h) Legs | <input type="checkbox"/> | <input type="checkbox"/> |
| i) Another part of the body not listed | <input type="checkbox"/> | <input type="checkbox"/> |
| j) I do not wear sun cream | <input type="checkbox"/> | <input type="checkbox"/> |

Q24 Generally how much time per day do you spend outside during daylight between **Monday** and **Friday** (e.g. working, running, playing sports)?

- | | | | | |
|-----------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| | < 1 hour | 1-3 hours | 4-6 hours | > 6 hours |
| a) April to September | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| b) October to March | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |

Q25 Generally how much time per day do you spend outside during daylight between **Saturday** and **Sunday** (e.g. working, running, playing sports)?

- | | | | | |
|-----------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| | < 1 hour | 1-3 hours | 4-6 hours | > 6 hours |
| a) April to September | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| b) October to March | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |

Q26 Do you regularly reapply sun cream while you are out in the sun?

Y N

Q27 (a) Do you regularly use sunbeds or tanning booths?

Y N

Q27 (b) If yes, how many times a month?



Appendix D Ambient conditions during sweat collection

November				May			
Sweat collection training session	Average temp (°C)	Average humidity (%)	Average wind speed (mph)	Sweat collection training session	Average temp (°C)	Average humidity (%)	Average wind speed (mph)
07 EB	24.7	27.2	In lab	07 HR	15	54	6
07 HB	24.7	27.2	In lab	08 HR	15	54	6
08 ER	1	98	3	10 HR	15	54	6
08 HR	7	91	9	11 HR	15	54	6
08 EB	Data missing			12 HR	15	54	6
08 HB	2.5	85	7.5	07 EB	13	78	8.5
09 ER	1	98	3	08 EB	14	78	8.5
09 HR	7	91	9	10 EB	15	78	8.5
09 EB	Data missing			11 EB	15	78	8.5
09 HB	2.5	85	7.5	12 EB	15	78	8.5
10 HR	7	91	9	14 EB	15	78	8.5
10 HB	2.5	85	7.5	07 ER	15	62	6
11 ER	1	98	3	08 ER	15	62	6
11 HR	7	91	9	10 ER	15	62	6
11 EB	Data missing			12 ER	15	62	6
11 HB	2.5	85	7.5	07 HB	20	57	15
12 ER	1	98	3	10 HB	20	57	15
12 HR	7	89	11	12 HB	20	57	15
12 EB	Data missing			14 HB	20	57	15
12 HB	2.5	85	7.5	09 HB	12.7	58	7
13 HR	7	91	9	13 HB	12.7	58	7
13 EB	Data missing			09 HR (track)	17	51.5	11.5
14 ER	1	98	3	13 HR (track)	17	51.5	11.5
14 HR	7	91	9	13 EB	12.5	84	10
14 EB	Data missing			09 EB	15.5	79.5	17
15 ER	10	82	10	Average	15.6	63.9	9.2
15 HR	7	73	12	SD	2.3	11.0	3.6
15 EB	5	90	20				
15 HB	11.5	87	18.5				
16 ER	17.9	67	In lab				
16 HR	7	73	12				
16 EB	11.5	79	7.5				
Average	7.1	82.9	8.6				
SD	6.6	18.3	4.4				

Appendix E Menstrual questionnaire

MENSTRUAL CYCLE DETAILS

(All information is fully confidential)

Please circle the answer where appropriate.

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 take any medication or hormones excluding contraception to regulate your menstrual cycle?

YES

NO

If YES, please state what you take and how often?

6) Do you take any other medication?

YES

NO

If YES, please state what you take and how often?

7) Have you previously used any form of hormonal contraception (oral contraceptive, implant, injection, coil)?

YES

NO

If YES, please state the type of contraception used and the date that you ceased using it?

If YES, when did you stop using it?

8) When did you have your last period (day 1)?

ORAL CONTRACEPTIVE DETAILS

(All information is fully confidential)

Please circle the answer where appropriate.

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 8 months?

YES

NO

6) Do you take any other medication?

YES

NO

If YES, please state what you take and how often?

Appendix F Example training log

(data taken from <https://www.trainingpeaks.com/>)

Weeks training log Sunday 8th November – Sat 14th November

Sunday

- 1 hour 32 minutes steady run (17.5km)

Monday

- 1 hour 30 swim (4.2km) hard speed session.
- 50 minute mobility gym (fairly easy)
- 30 minute steady cycle
- 1 hour run (10km) hard run with hill sprints

Tuesday

- 1 hour 17 minute swim (4650m, strength session)
- 1 hour 30 minute bike steady
- 35 minute easy run (6km)
- 1 hour 15 minute run , easy (13km)

Wednesday

- 1 hour 30 swim (4.5km long steady reps relatively hard)
- 2 hour 30 ride (steady)
- 1 hour hilly run (10km)

Thursday

- 1 hour 30 swim (4.6km strength set)
- 2 hour mountain bike (25km will have hard uphill efforts)
- 1 hour 7 minute run (steady 14km)

Friday

- 1 hour easy swim (3km technique)
- 3 hour 40 minute steady ride
- 1 hour all round gym
- 45 minute easy run (7km)

Saturday

- Hard 1 hour treadmill run (with hard efforts)
- Easy 15 minute cool down run
- Harder 1 hour 30 swim (4.8km)
- 3 hours 30 minute steady ride.

Appendix G Example diet log

Thursday 5th May

Breakfast pre-swim – 1 cup of Scottish porridge oats, 260 ml SS milk, 1 teaspoon honey (pure clear squeezy)

During swim – 750 ml squash no added sugar

Post-swim (10:40am) – 2 slices of Kingsmill thick 50/50 bread, one with Norpack slightly salted butter and tesco strawberry jam and one with butter and light cover of crunchy peanut butter

11:00 – Nature valley protein bar roasted peanuts and pumpkin seeds 30g

During ride – 750 ml orange squash with 1 maxifuel viper electrolyte tablet (drank about half)

2:00 pm – 1 banana 237g whole (straight after ride)

Lunch (2:20pm) – 2 slices Kingsmill 50/50 bread toasted, 2 large eggs scrambled with 50 ml of semi skimmed milk and teaspoon extra virgin olive oil, 1 slice tesco finest crumbed Wiltshire cured ham (30g), few drops of lea and Perrins Worcestershire sauce and 1 tablespoon of HP sauce, 150 ml smooth orange juice from concentrate

1 pot of Petit Filous fromage frais (85g)

1 tesco finest roasted hazelnut oats and chocolate cookie 72g

Post-run (5:40) – 1 braeburn apple (149g whole)

2 tesco raisin and lemon pancakes toasted with butter (35g each)

Dinner – slow cooked beef casserole with rice (easy cook long grain 117g),

Total recipe – casserole beef steak (402 g), 2 peppers (1 yellow 1 red) 315g, tin of chunky chopped tomatoes (400g), 1 red onion 84g, carrots 63g, 2 tablespoons of tomato puree.
Only half of this eaten.

8:30 – ½ cookie (47g)

Friday 6th May

Breakfast (8:00) – 1 cup mornflake superfrost oats, 260 ml SS milk, 1 teaspoon of honey, 28g whole almonds, 180 ml smooth orange juice from concentrate

Post-run (11:15am) – powerbar 30% protein plus bar 55g chocolate flavour

11:45 – 180 ml SS milk, 2 lemon and raisin pancakes toasted with butter (35g each)

1:20 – 96g red seedless grapes, Nature valley protein bar (30g) same flavour

Lunch (1:30) – 2 slices Kingsmill 50/50 bread, 2 slices tesco finest crumbed Wiltshire cured ham (30g each). 32g aldi mature cheddar cheese, 2 teaspoons of tesco sandwich pickle, 1 pot petit filous fromage frais (85g)

16:00 – 129g malted weaties with 240 ml SS milk, tesco thick and creamy raspberry yoghurt (100g), 1 tesco suntrail farms plum (78g whole)

18:00 – 240 ml orange juice from concentrate

Dinner (20:00) – ½ packet Schwartz spaghetti carbonara packet mix (32g whole packet), 250ml SS milk, 1 chicken breast (150g) fried with splash of extra virgin olive oil, 180g uncooked pasta, 82g courgette

1 Mr Kipling apple and blackcurrent pie (59g) and 39g Malteasters chocolate

20:45 – cup of tea with SS milk

Saturday 7th May

Breakfast (8:00) – 1 cup Mornflake superfrost oats, 260ml SS milk, 40g whole almonds, 1 teaspoon of honey, 240 ml smooth orange juice from concentrate

11:30 – Nature valley protein bar (30g) during ride, 2 Hovis crumpets with butter and strawberry jam (60g each), 1 tesco finest roasted hazelnut, oats and chocolate cookie (60g)

Lunch (12:45) – sweet potato 224g cooked in microwave with 263g beef casserole from last night about ¼ total

3:45pm – 1 tesco suntrail farms plus (74g), 102g red seedless grapes

5:45pm – 71g cheerios with 240 ml SS milk, 2 slices Kingsmill 50/50 bread toasted with Norpack butter, one with strawberry jam and the other with crunchy peanut butter

Dinner (20:00) – Lasagne (cooked the other night) 364g eaten and it contained Aldi lean mince beef, onion, carrots, courgette, curly kale, Aldi red + white sauce, mature cheddar cheese

240 ml apple juice from concentrate

Petit filous fromage frais (85g)

9:15 pm – 40g Teasers chocolate

Sunday 8th May

Breakfast (7:45) – 2 slices of Kingsmill 50/50 bread with butter and tesco strawberry jam, banana (208g with skin), 240 ml apple juice

Post-run (10:15) – 1 cup mornflake oats, 260 ml SS milk, 33g whole almonds, 240 ml OJ from concentrate

12:15 – petit filous yog same as before

Lunch (15:00) – banana (249g), tesco thick and creamy strawberry yoghurt (100g), 98g uncooked pasta with chicken breast fillet (91g) fried with a splash of extra virgin olive oil, cathedral city mature cheddar cheese (42g grated)

1 mr kipling apple and blackcurrent pie (59g)

18:00 – 2 Hovis crumpets both with thick spread of tesco strawberry jam (60g each), cup of tea with SS milk

19:00 – 88g red seedless grapes, 61g cheerios with 240 ml SS milk, 40g Teasers chocolate

Dinner (19:45) – sweet potato (190g) peeled, chopped and mashed with a splash of SS milk, 120g aldi salmon fillet, 60g carrots, 40g peas

Monday 9th May

Breakfast pre-swim – 2 slices Kingsmill 50/50 bread with thick spread of tesco strawberry jam

8:15 – 1 cup mornflake oats, 260 ml SS milk, 27g whole almonds, banana (199g), 240 ml OJ from concentrate

10:40 – tesco thick and creamy strawberry yoghurt (100g), Nature valley protein bar 30g

Lunch (13:00) – plum (80g), 2 slices normal bread with 42g cathedral city mature cheddar cheese and 2 teaspoons of tesco sandwich pickle

14:30 – 57g cheerios with 180 ml SS milk, 69g red seedless grapes, braeburn apple (148g whole)

16:00 – 2 Hovis crumpets with Bertolli spread, 3 Fox's chocolatey milk chocolate rounds (17g each)

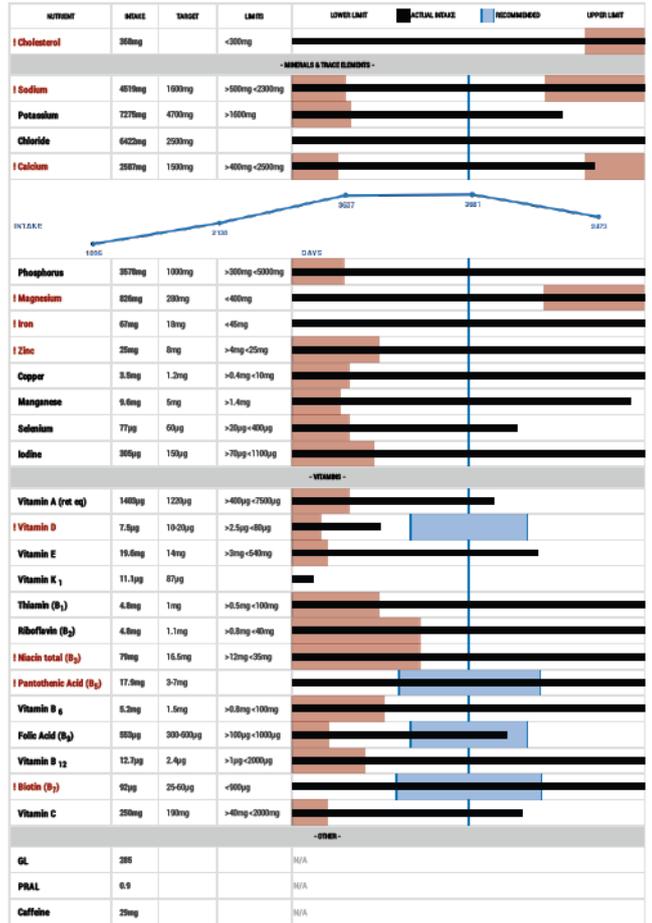
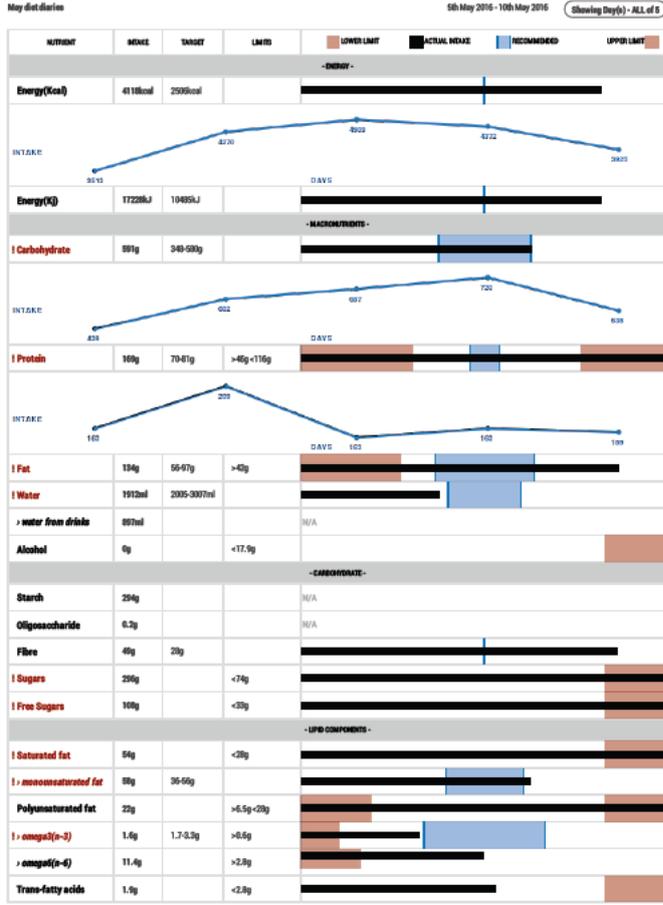
19:00 – 400ml SS milk straight after bike session

Dinner (20:00) – sausage and bean casserole (slow cooker) with mashed potato (197g)

Contained 400g chunky chopped tomatoes, 400g cannellini beans in water, 2 tomato puree, 400g tesco finest pork sausages, onion (61g). Ate about half of this.

1 mr kipling apple and blackcurrent pie (59g)

Appendix H Example Nutritics analysis



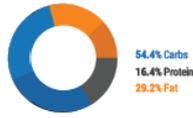
NUTRIENT	MEASURE	TARGET	LIMITS	LOWER LIMIT	ACTUAL MEAS	RECOMMENDED	UPPER LIMIT
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Figures from Health guidelines for female a 17-65 years old

Generated by Nutritics v4.2.5 on 2nd Aug 2016

Macronutrient Analysis

	CARBOHYDRATE	PROTEIN	FAT	ALCOHOL
Intake	591.3g	169.2g	133.6g	0g
g/kg body-weight	10.2	2.9	2.3	0
KiloCal	2238	677	1202	0
KiloCal %	54.4%	16.4%	29.2%	0%



DAY 5

- Oil, flaxseed, cold-pressed, average 85g
- Milk, semi-skimmed, pasteurized, average 845g
- Bread, white, with added flour, toasted 100g
- Jam, fruit with added seeds 30g
- Nature Valley Protein bar with peanuts, pumpkin and sunflower seeds 30g
- Bananas, raw, fresh only, weighed with skin 199g
- Orange juice, fresh concentrate 245g
- Apples, eating raw, fresh and skin 148g
- Almonds, with skin 27g
- Oranges, red 60g
- Cheese, Cheddar, English 43g
- Bransford Protein Original 20g
- Yogurt, virtually fat free/lowfat, fruit 100g
- Plums, average, raw, fresh and skin 85g
- Pie, fruit, individual, retail 50g
- Breakfast cereal, multigrain hoops, fortified 120g
- Cranquets, toasted 74g
- Bread, brown, average 10g
- Fat spread, low fat (20-25%), not polyunsaturated, with olive oil 15g
- Biscuits, soft, butter, chocolate, half coated 48g
- Casomile, sausage, homemade 400g
- Peanut oil, matched with butter 100g

Notes

Diet Log

DAY 1	DAY 2	DAY 3	DAY 4	DAY 5
Oil, flaxseed, cold-pressed, average 85g	Milk, semi-skimmed, pasteurized, average 845g	Oil, flaxseed, cold-pressed, average 85g	Milk, semi-skimmed, pasteurized, average 845g	Oil, flaxseed, cold-pressed, average 85g
Milk, semi-skimmed, pasteurized, average 845g	Bread, white, with added flour, toasted 100g	Milk, semi-skimmed, pasteurized, average 845g	Bread, white, with added flour, toasted 100g	Milk, semi-skimmed, pasteurized, average 845g
Bread, white, with added flour, toasted 100g	Jam, fruit with added seeds 30g	Bread, white, with added flour, toasted 100g	Jam, fruit with added seeds 30g	Bread, white, with added flour, toasted 100g
Jam, fruit with added seeds 30g	Nature Valley Protein bar with peanuts, pumpkin and sunflower seeds 30g	Nature Valley Protein bar with peanuts, pumpkin and sunflower seeds 30g	Nature Valley Protein bar with peanuts, pumpkin and sunflower seeds 30g	Nature Valley Protein bar with peanuts, pumpkin and sunflower seeds 30g
Nature Valley Protein bar with peanuts, pumpkin and sunflower seeds 30g	Bananas, raw, fresh only, weighed with skin 199g	Nature Valley Protein bar with peanuts, pumpkin and sunflower seeds 30g	Bananas, raw, fresh only, weighed with skin 199g	Nature Valley Protein bar with peanuts, pumpkin and sunflower seeds 30g
Bananas, raw, fresh only, weighed with skin 199g	Orange juice, fresh concentrate 245g	Bananas, raw, fresh only, weighed with skin 199g	Orange juice, fresh concentrate 245g	Bananas, raw, fresh only, weighed with skin 199g
Orange juice, fresh concentrate 245g	Apples, eating raw, fresh and skin 148g	Orange juice, fresh concentrate 245g	Apples, eating raw, fresh and skin 148g	Orange juice, fresh concentrate 245g
Apples, eating raw, fresh and skin 148g	Almonds, with skin 27g	Apples, eating raw, fresh and skin 148g	Almonds, with skin 27g	Apples, eating raw, fresh and skin 148g
Almonds, with skin 27g	Oranges, red 60g	Almonds, with skin 27g	Oranges, red 60g	Almonds, with skin 27g
Oranges, red 60g	Cheese, Cheddar, English 43g	Oranges, red 60g	Cheese, Cheddar, English 43g	Oranges, red 60g
Cheese, Cheddar, English 43g	Bransford Protein Original 20g	Cheese, Cheddar, English 43g	Bransford Protein Original 20g	Cheese, Cheddar, English 43g
Bransford Protein Original 20g	Yogurt, virtually fat free/lowfat, fruit 100g	Bransford Protein Original 20g	Yogurt, virtually fat free/lowfat, fruit 100g	Bransford Protein Original 20g
Yogurt, virtually fat free/lowfat, fruit 100g	Plums, average, raw, fresh and skin 85g	Yogurt, virtually fat free/lowfat, fruit 100g	Plums, average, raw, fresh and skin 85g	Yogurt, virtually fat free/lowfat, fruit 100g
Plums, average, raw, fresh and skin 85g	Pie, fruit, individual, retail 50g	Plums, average, raw, fresh and skin 85g	Pie, fruit, individual, retail 50g	Plums, average, raw, fresh and skin 85g
Pie, fruit, individual, retail 50g	Breakfast cereal, multigrain hoops, fortified 120g	Pie, fruit, individual, retail 50g	Breakfast cereal, multigrain hoops, fortified 120g	Pie, fruit, individual, retail 50g
Breakfast cereal, multigrain hoops, fortified 120g	Cranquets, toasted 74g	Breakfast cereal, multigrain hoops, fortified 120g	Cranquets, toasted 74g	Breakfast cereal, multigrain hoops, fortified 120g
Cranquets, toasted 74g	Bread, brown, average 10g	Cranquets, toasted 74g	Bread, brown, average 10g	Cranquets, toasted 74g
Bread, brown, average 10g	Fat spread, low fat (20-25%), not polyunsaturated, with olive oil 15g	Bread, brown, average 10g	Fat spread, low fat (20-25%), not polyunsaturated, with olive oil 15g	Bread, brown, average 10g
Fat spread, low fat (20-25%), not polyunsaturated, with olive oil 15g	Biscuits, soft, butter, chocolate, half coated 48g	Fat spread, low fat (20-25%), not polyunsaturated, with olive oil 15g	Biscuits, soft, butter, chocolate, half coated 48g	Fat spread, low fat (20-25%), not polyunsaturated, with olive oil 15g
Biscuits, soft, butter, chocolate, half coated 48g	Casomile, sausage, homemade 400g	Biscuits, soft, butter, chocolate, half coated 48g	Casomile, sausage, homemade 400g	Biscuits, soft, butter, chocolate, half coated 48g
Casomile, sausage, homemade 400g	Peanut oil, matched with butter 100g	Casomile, sausage, homemade 400g	Peanut oil, matched with butter 100g	Casomile, sausage, homemade 400g
Peanut oil, matched with butter 100g		Peanut oil, matched with butter 100g		Peanut oil, matched with butter 100g

Appendix I Example 48 h lead in diet

Please follow this diet as closely as possible 48 hours before each main trial. Please remain fasted from 20:00 the night before a trial, do not consume breakfast but drink 500ml of plain water when you wake up before arriving at the lab.

Consume meals and snacks at normal times (times for guidance based on your 3 day diet logs)

Day 1

Breakfast (7:30 am)

Coco pops 80g

Semi skimmed milk 200 ml

Lunch (12:30 pm)

English muffins toasted x 2

Flora spread lightly

2 large eggs

Dinner (7:00 pm)

White rice 110g

Naan bread 100g

Pepper 100g

Red onion 80g

Onion 80g

Chicken breast 140g

Lloyd Grossman Balti sauce 170g

Snacks

Robinsons sugar free squash – normal amount

Wholemeal rolls x 3

Parma ham x 5 slices

Cheese twists 80g

Rock cakes x 2

Day 2

Breakfast (7:30 am)

Wholemeal toast x 2 slices

Flora spread lightly

Lunch (12:30 pm)

Wholegrain rolls x 2

Parma ham x 2 slices

Dinner (7:30 pm)

Fresh pasta 200g

Pancetta 50g

Double cream 75ml

3 eggs

Snacks

Robinsons sugar free squash – normal amount

Wholemeal rolls x 2

Parma ham x 2 slices

Cheese twists 80g

Cherry scones x 2

Flapjack 60g

Appendix J Supplement screening certificate

UKAS Testing Laboratory No: 1187



Nathan Lewis
English Institute of Sport
Sports Training Village
University of Bath
Claverton Down
Bath
BA2 7AY
UK

LGC
Newmarket Road
Fordham
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UK

Tel: +44 (0)1638 720500
Fax: +44(0)1638 724200
Email: info@lgcgroup.com
www.lgcgroup.com

Date Issued: 23 July, 2014

CERTIFICATE OF ANALYSIS: 91530

LGC Supplement Screen

Consignment Number:	Post
Delivery Date:	10 July, 2014
Date Analysis Commenced:	10 July, 2014
Purchase Order Number:	N/A

Product:	Impact Whey Isolate & Dextrose Mix	Programme Category:	Routine
Flavour:		Pack Size:	
Batch No:	57252/57124	LGC Reference:	694023
Batch Expiry:	5/2015		

The sample was analysed using documented LGC screening methods for the compounds specified within the Service Level Agreement: Nutritional Supplements V2.0.

GCMS:
None were found.

LCMS:
None were found.

Signed


Kate Marrs
Senior Scientist

Test results apply to the portion of product taken.
* or isomers of - as specified within the service level agreement.

Appendix K Supplement disclaimer

As part of this study you will be consuming a supplement. If you are an elite sportsperson i.e. international or national standard who may undergo either out-of and in-competition (or both) doping tests it is important that you consider the following:

- 1) The supplement being studied could be contaminated with a substance that appears on the banned list. There is evidence from research that around 15% of supplements can be contaminated accidentally with prohormones of testosterone and nandrolone ¹. Even well-known brands from the UK and USA have been found to be contaminated.
- 2) You are responsible for what goes into your body and unless it can be guaranteed that what you take is “clean” then you should not take it.

Participant Name (Please print): _____

Signature: _____

Date: _____

Reference:

1) *H.Geyer et al.* [Int J Sports Med.](#) 2004 Feb;25(2):124-9 Analysis of non-hormonal nutritional supplements for anabolic-androgenic steroids - results of an international study.

Appendix L Standard operating procedure for the production of the CHO+PRO recovery solutions

This SOP was prepared prior to the start of the study, to ensure that the solutions were made in the same way each time and were made in a clean, contamination free environment.

Who will prepare the drinks?

Primary responsibility will lie with the main investigator (Becky Townsend). The importance of avoiding contamination will be fully explained to any undergraduate students that may assist with the study and they will not be allowed to handle the products or equipment unless the main investigator sees appropriate.

Where will the products and equipment be stored?

Drink preparation will take place in 138 ERD which is the designated area for food preparation.

All batch tested products including; whey protein isolate, glucose (dextrose), sweetener and flavouring drops and equipment including; a blender, bottles/shakers (different bottles used for active and placebo drinks), measuring jugs/cylinders, weighing scales, spoons/scoops, sterilising solution (Alcohol), washing up bowl, towels, will be kept separate from all other equipment in a secured cupboard to prevent use for other purposes.

No lab coats or any lab equipment is to be taken into the kitchen.

Preparation procedure:

1. After thoroughly cleaning hands (especially after touching any lab equipment) and surfaces, appropriate amounts of products will be transferred from sealed bags into bottles using a spoon and weighed with electronic scales.
2. Appropriate volumes of water (tap water) will be measured using a measuring jug/cylinder and transferred into the bottles.
3. The solution will then be transferred to the blender for mixing and then back to the bottles for consumption.
4. Labelled bottles will be kept in the fridge in the kitchen until the trial begins.

Cleaning procedure:

1. Once drink preparation is complete, all used equipment (including the blender) will be cleaned thoroughly in a washing up bowl with washing up liquid and sponges.
2. Equipment will be dried using towels and placed in the secure cupboard.
3. The same will be done for the bottles once the trial is complete.

What happens in the case of contamination?

1. If contamination occurs from lab equipment being brought into the kitchen or the equipment being used for other purposes, all surfaces will be cleaned using disinfectant and equipment sterilised using Milton solution (or similar).
2. The batch tested products **MUST NOT** leave the kitchen or be touched/used by anyone other than the main investigator. As contamination of the products by potentially banned/illegal or harmful substances will mean the products can no longer be used.

Taste matching:

- Both the CHO+PRO drink and the placebo drink were taste matched using artificial flavourings (raspberry, vanilla or apple) and sucralose was added to the placebo drink to match the sweetness of the glucose added to the CHO+PRO drink. 15 flavour drops were added to each drink and around 1 small scoop of sucralose was needed in the placebo drink. Drinks were made the evening before testing to allow the protein to 'settle' in the CHO+PRO

drinks; otherwise the drink was less palatable and would not fit into a single bottle. Bottles were covered to ensure that participants were unaware of which drink they were consuming.

- To test the taste matching, both drinks were given to 6 participants in the laboratory. On 3 different occasions (to test the 3 different flavours), a CHO+PRO and a placebo drink were provided in covered bottles which participants consumed in any order. All 6 of the participants were unable to tell the difference between the CHO+PRO and the placebo drinks in all 3 trials.

Appendix M Example experimental diet

Food	Energy (kcal)	CHO (g)	PRO (g)	Fat (g)
Recovery solution				
PRO (whey isolate) 29 g	107	0	27	0
CHO (dextrose) 87 g	315	78	0	0
Sub total	422	78	27	0
Evening meal and snacks				
Beef lasagne 400 g	551	47	31	25
½ Garlic baguette	272	32	6	13
Cheddar cheese 25 g	98	1	6	8
Chocolate sponge pudding	261	38	3	11
Rice Krispies square cereal bar 36 g	158	26	2	5
Chocolate chip and nut cereal bar	91	14	1	3
Banana (medium size)	146	39	1	0
Sub total	1,577	197	50	65
Total	1,999	276	77	65
Diet composition (%)		55	15	30

Appendix N Parathyroid Hormone Secretion is Controlled by Both Ionised Calcium and Phosphate During Exercise and Recovery in Men

Rebecca Townsend^{1,5}, Kirsty J. Elliott-Sale¹, Ana Jessica Pinto², Craig Thomas¹, Jonathan P.R. Scott^{3,4}, Kevin Currell⁵, William D. Fraser⁶, Craig Sale¹

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⁵English Institute of Sport, EIS Performance Centre, Loughborough University, UK.

⁶Norwich Medical School, University of East Anglia, UK, Norfolk and Norwich University Hospital Norfolk UK.

Abbreviated title: PTH Regulation During Exercise and Recovery

Key words: PTH, Exercise, Recovery, Calcium, Phosphate

Word count: after addressing reviewers comments = 4, 028

Number of figures and tables: 5

Corresponding author: Professor Craig Sale, Musculoskeletal Physiology Research Group, Sport, Health and Performance Enhancement Research Centre, School of Science and Technology, Nottingham Trent University, NG11 8NS, UK. E-mail: craig.sale@ntu.ac.uk.

Disclosure statement: The authors have nothing to disclose

Abstract

The mechanism by which PTH is controlled during and after exercise is poorly understood due to insufficient temporal frequency of measurements.

Objective

To examine the temporal pattern of PTH, PO₄, ACa and Ca²⁺ during and after exercise.

Design and setting

A laboratory-based study with a cross-over design, comparing 30 min of running at 55%, 65% and 75% VO_{2max}, followed by 2.5-h of recovery. Blood was obtained at baseline, after 2.5, 5, 7.5, 10, 15, 20, 25 and 30 min of exercise and after 2.5, 5, 7.5, 10, 15, 20, 25, 30, 60, 90 and 150 min of recovery

Participants

Ten men (age 23±1 y, height 1.82±0.07 m, body mass 77.0±7.5 kg) participated.

Main Outcome Measures

PTH, PO₄, ACa and Ca²⁺

Results

Independent of intensity, PTH concentrations decreased with the onset of exercise (-21 to -33%; $P\leq 0.001$), increased thereafter and were higher than baseline by the end of exercise at 75%VO_{2max} (+52%; $P\leq 0.001$). PTH peaked transiently after 5–7.5 min of recovery (+73 to +110%; $P\leq 0.001$). PO₄ followed a similar temporal pattern to PTH and Ca²⁺ followed a similar but inverse pattern to PTH. PTH was negatively correlated with Ca²⁺ across all intensities ($r=-0.739$ to -0.790 ; $P\leq 0.001$). When PTH was increasing, the strongest cross-correlation was with Ca²⁺ at 0 lags (3.5 min) ($r=-0.902$ to -0.950); during recovery, the strongest cross-correlation was with PO₄ at 0 lags (8 min) ($r=0.987$ to 0.995).

Conclusions

PTH secretion during exercise and recovery is controlled by a combination of changes in Ca²⁺ and PO₄ in men.

Abbreviations

ACa, albumin-adjusted calcium; Ca, calcium; Ca²⁺, ionised calcium; CV, coefficient of variation; PO₄, phosphate; PTH, parathyroid hormone; VO_{2max}, maximal oxygen consumption.

Introduction

At rest, PTH secretory activity is regulated by serum ionised calcium (Ca^{2+}), which is detected by the calcium-sensing receptor on the chief cells of the parathyroid gland (1). When Ca^{2+} decreases from the homeostatic set point, PTH is synthesised and secreted, increasing serum calcium (Ca) through mobilisation of the bone reservoir via bone resorption, and by increasing renal tubular reabsorption and intestinal Ca absorption (2–4). PTH has a dual effect on bone that appears to be dependent on the signalling mechanism and the length of time that concentrations remain elevated for (5). Prolonged elevations in PTH, that are seen with endurance type exercise, and that can also result in the loss of the circadian rhythm of PTH, might cause an increase in bone resorption, whereas, transient spikes in PTH, that are seen with high intensity interval type training, might cause an increase in bone formation (6), provided that the magnitude of the increase is sufficient. Chronic elevations in PTH concentrations have been associated with increased fracture risk (7, 8). Complete fractures and stress fractures are also debilitating injuries for elite athletes (9), therefore understanding how PTH is regulated during exercise and recovery may have implications for both the general population and athletes who are at risk of chronically elevated PTH concentrations, as a positive calcium balance is necessary for bone adaptation to mechanical loading (10).

Exercise increases PTH concentrations (11–20), although studies have used different exercise modes, durations and intensities. Exercise intensity is important, given that Scott *et al.* (17) have shown that 60 min of running at 55%, 65% and 75% of maximal oxygen consumption ($\text{VO}_{2\text{max}}$) results in different PTH responses during and after exercise. Any study investigating the underlying mechanisms responsible for the changes in PTH during exercise and recovery should examine the effects of exercise intensity.

During exercise, reductions in circulating Ca do not explain the increase in PTH, as the concentration of albumin-adjusted calcium (ACa) – a surrogate for Ca^{2+} – is either increased (12, 15, 17) or unchanged (14, 18, 19) concomitantly with PTH. Barry *et al.* (16) showed that Ca ingestion before exercise attenuated, but did not abolish the increase in PTH, suggesting that some other mechanism

contributed to the increase. This could involve phosphate (PO_4), as an increase in PO_4 increases PTH in rested individuals (21). Following exercise, PO_4 concentrations decrease and the timing and magnitude of these decreases reflect those in PTH (17, 18, 20), also suggesting that PO_4 may be involved in PTH regulation with exercise.

The hypothesis that decreased Ca^{2+} triggers increased PTH during exercise has not yet been proven (16). PTH is secreted within seconds of a decrease in Ca^{2+} and subsequent increases in Ca^{2+} take only minutes to occur in response to increased PTH, highlighting a dynamic relationship (1, 22). Despite this, no studies have measured PTH and other markers of Ca metabolism until 20 minutes of exercise has been completed, by which time PTH is elevated. Most studies have started taking measurements at 30 min post-exercise, by which time PTH has returned to near pre-exercise levels (15–19, 23). Single or infrequent measurements of PTH, ACa and PO_4 during and after exercise might fail to capture the dynamic nature of Ca regulation with exercise (16). Using repeated measurements with a high frequency, we examined the temporal pattern of PTH, PO_4 , ACa and Ca^{2+} during and after 30 minutes of treadmill running at three exercise intensities.

Materials and Methods

Participants

Ten healthy, physically active men ([mean±SD] age 23±1 y, height 1.82±0.07 m, body mass 77.0±7.5 kg) volunteered for the study, which was approved by the Institutional Ethics Committee. Participants were non-smokers, had not suffered a fracture in the past 12 months, were free from musculoskeletal injury and were not taking any medication or experiencing any problems known to affect Ca or bone metabolism. Eligibility was confirmed during the initial session, when participants provided written informed consent.

Experimental Design

Participants completed a preliminary visit for health screening, habituation and measurement of VO_{2max} . Participants then completed three randomised (Latin Square Design), three-day experimental trials, each separated by one week. On days 1–2, participants refrained from exercise, caffeine and alcohol. On day 2, participants consumed a self-selected diet that was repeated for each trial. On day 3, participants performed a 30 min bout of running at 55%, 65% and 75% VO_{2max} , followed by 2.5 h of recovery.

Trial Procedures

VO_{2max}

Participants performed an incremental treadmill test to determine lactate threshold, followed by a ramp test to determine VO_{2max} , as per Jones and Doust (24). The level running velocities corresponding to 55% ($8.7±0.6$ km·h⁻¹), 65% ($10.1±0.8$ km·h⁻¹) and 75% VO_{2max} ($11.9±0.9$ km·h⁻¹) were calculated based on the regression of VO_2 and velocity.

Main Trials

Participants arrived (09:00) following an overnight fast and after consuming 500 mL of water upon awakening. After voiding, participants had their body mass measured before adopting a semi-recumbent position and having a cannula inserted into a forearm vein. After 10 min rest, a baseline blood sample (5 mL) was collected for measurement of PTH, PO₄, ACa and Ca²⁺. Thirty min of treadmill running at 55%, 65% or 75% VO_{2max} commenced thereafter. Additional blood was collected after 2.5, 5, 7.5, 10, 15, 20, 25 and 30 min of exercise. After exercise, participants adopted a semi-recumbent position and blood was collected at 32.5, 35, 37.5, 40, 45, 50, 55, 60, 90, 120 and 180 min. Ca²⁺ was measured immediately but due to equipment availability Ca²⁺ was only measured in participants 5–10. Blood samples were transferred to pre-cooled standard serum tubes (Becton Dickinson Vacutainer System, USA) to clot at room temperature for 60 min. Samples were centrifuged at 2000 rev·min⁻¹ and 5°C for 10 min and the resulting serum was transferred into Eppendorf tubes and frozen at -80°C. Following the last blood sample, the cannula was removed and body mass measured. Participants were given 3 mL·kgBM⁻¹·h⁻¹ of water to consume throughout the trials. The timings of blood samples and exercise were identical in each trial to ensure that circadian rhythms of the metabolites were controlled for.

Biochemical Analysis

PTH was measured using ECLIA on a Modular Analytics E170 analyser (Roche Diagnostics, Burgess Hill, UK). Inter-assay CV for PTH was <4% between 1–30 pmol·L⁻¹ and sensitivity of 0.8 pmol·L⁻¹. PO₄, total Ca and albumin were measured using standard colorimetric assays and spectrophotometric methods, performed on an ABX Pentra 400 (Horiba ABX, Montpellier, France). Inter-assay CVs were ≤3.6% between 0.09–7.80 mmol·L⁻¹ for PO₄, ≤1.7% between 0.04–5.00 mmol·L⁻¹ for total Ca and ≤1.9% between 0.02–5.99 g·dL⁻¹ for albumin. Because fluctuations in protein, particularly albumin, may cause total Ca levels to change independently of the Ca²⁺ concentrations, total Ca concentrations were corrected to give albumin-adjusted Ca values: 0.8 mg·dL⁻¹ was subtracted from total Ca concentrations for every 1.0 g·dL⁻¹ that albumin concentrations were less than 4 g·dL⁻¹ or 0.8 mg·dL⁻¹ was added to total Ca concentrations for every 1.0 mg·dL⁻¹ that albumin concentration were greater than 4 mg·dL⁻¹. Ca²⁺, glucose and lactate were

measured in whole blood using a blood gas analyser (Radiometer ABL90 FLEX, Copenhagen, Denmark). Ca^{2+} is estimated directly between pH 7.2-7.6 with no pH correction applied. The inter- and intra-assay CV for Ca^{2+} was $\leq 3\%$ between 0.2–9.99 $\text{mmol}\cdot\text{L}^{-1}$, for glucose was $\leq 5\%$ between 0–60 $\text{mmol}\cdot\text{L}^{-1}$ and for lactate was $\leq 26.7\%$ between 0.1–31 $\text{mmol}\cdot\text{L}^{-1}$.

Statistical Analysis

Statistical significance was accepted at $P \leq 0.05$. Baseline concentrations were compared using one-way ANOVA. All data were analysed using repeated measures ANOVA, with *Intensity* (55% vs 65% vs 75% $\text{VO}_{2\text{max}}$) and *Time* (of sampling) as within subject factors. Parametric assumptions of normality and sphericity were confirmed using Shapiro-Wilks and Mauchly's tests. Tukey's HSD *post-hoc* test was used to compare timepoints against baseline and to compare exercise intensities at each timepoint, where appropriate. Pearson's correlation coefficients were calculated for PO_4 , ACa and Ca^{2+} with PTH.

Cross-correlational analyses were performed to determine the temporal relationships between PTH and PO_4 , ACa and Ca^{2+} . Cubic interpolation was performed to adjust for unevenly spaced data points and cross-correlational analyses were subsequently performed using R (version 3.2.2, Vienna, Austria). To determine whether one time series led another, cross-correlation functions were computed at seven lag time points for 'PEAK' (data points between baseline and peak PTH concentrations [5 min of recovery]), where each lag represented 3.5 min, and six lag time points for 'DEC' (all data points during the decrease in PTH concentrations [5 to 90 min of recovery]), where each lag represented 8 min.

Results

Baseline biochemistry

Baseline PTH, PO₄, Aca and albumin were not significantly different between trials ($P=0.339$ to 0.982). Baseline Ca²⁺ at 55% VO_{2max} was significantly ($P\leq 0.05$) higher than at 65% VO_{2max} and 75% VO_{2max} (Table 1).

PTH

There was no main effect of *Intensity*, but there was a main effect of *Time* ($P\leq 0.001$) and an *Intensity* \times *Time* interaction ($P\leq 0.001$). PTH concentrations decreased with the onset of exercise and were significantly lower than baseline after 5 min of exercise at 55% VO_{2max} (-23%; $P\leq 0.05$) and 75% VO_{2max} (-33%; $P\leq 0.001$), but not at 65% VO_{2max} (-21%; $P=0.305$) (Fig. 1A all participants; Fig. 2A participants 5–10). Thereafter, PTH increased, becoming significantly greater than baseline at the end of exercise (30 min) at 75% VO_{2max} (+52%; $P\leq 0.001$) and after 2.5 min of recovery at 55% VO_{2max} (+43%; $P\leq 0.001$) and 65% VO_{2max} (+52%; $P\leq 0.001$). PTH concentrations peaked after 5 min of recovery at 55% VO_{2max} (+73%; $P\leq 0.001$) and 75% VO_{2max} (+110%; $P\leq 0.001$), and after 7.5 min of recovery at 65% VO_{2max} (+76; $P\leq 0.001$). PTH concentrations then decreased, but remained significantly higher than baseline until 15 min into recovery at 55% VO_{2max} and until 25 min at 65% VO_{2max} and 75% VO_{2max}. PTH concentrations decreased below baseline after 60 min of recovery in all trials (-8% to -17%).

PTH concentrations were not significantly different at any time point between 55% and 65% VO_{2max} trials. Exercise at 75% VO_{2max} resulted in significantly higher PTH concentrations than at 55% VO_{2max} at the end of exercise ($P\leq 0.001$), and at 2.5 ($P\leq 0.001$), 5 ($P\leq 0.001$), 7.5 ($P\leq 0.05$), 10 ($P\leq 0.05$) and 15 ($P\leq 0.001$) min into recovery, and higher than exercise at 65% VO_{2max} at the end of exercise ($P\leq 0.001$), and at 2.5 ($P\leq 0.001$) and 5 ($P\leq 0.001$) min into recovery.

PO₄

There was no main effect of *Intensity*, but there was a main effect of *Time* ($P \leq 0.001$) and an *Intensity* \times *Time* interaction ($P \leq 0.05$). PO_4 concentrations increased with the onset of exercise at all intensities, being significantly higher than baseline from 7.5 min to the end of exercise at 55% VO_{2max} (+16%; $P \leq 0.001$), and between 5 min and the end of exercise at 65% VO_{2max} (+22%) and 75% VO_{2max} (+26%) ($P \leq 0.05$ to $P \leq 0.001$) (Fig. 1B). PO_4 concentrations peaked at the end of exercise, and decreased thereafter, but remained significantly higher than baseline until 5 min into recovery at 55% VO_{2max} , 10 min at 65% VO_{2max} and 15 min at 75% VO_{2max} . PO_4 concentrations decreased below baseline at 60 min of recovery and remained so until 150 minutes of recovery at 65% VO_{2max} (-5 to -10%) and 75% VO_{2max} (-7 to -12%) ($P \leq 0.05$ to $P \leq 0.001$). Concentrations did not decrease significantly below baseline at 55% VO_{2max} .

Exercise at 65% VO_{2max} resulted in significantly higher PO_4 concentrations than exercise at 55% VO_{2max} at 10 ($P \leq 0.05$), 20 ($P \leq 0.001$) and 25 ($P \leq 0.05$) min of exercise.

ACa

There was no main effect of *Intensity*, but there was a main effect of *Time* ($P \leq 0.001$) and an *Intensity* \times *Time* interaction ($P \leq 0.001$). ACa concentrations increased with the onset of exercise and were significantly higher than baseline between 7.5 min and the end of exercise at 65% VO_{2max} (+9%; $P \leq 0.001$) and between 2.5 min and the end of exercise at 75% VO_{2max} (+14%; $P \leq 0.001$) (Fig. 1C). ACa concentrations peaked after 20 min of exercise and decreased thereafter, but remained significantly higher than baseline until 5 min into recovery at 65% VO_{2max} and 7.5 minutes at 75% VO_{2max} . ACa concentrations decreased below baseline 15 min into recovery and remained so until 30 min of recovery at 55% VO_{2max} (-7 to -9%; $P \leq 0.05$ to $P \leq 0.001$). Concentrations decreased below baseline 25 min into recovery and remained so until 90 min of recovery at 65% VO_{2max} (-6 to

-8%; $P \leq 0.05$ to $P \leq 0.001$). A Ca concentrations did not decrease significantly below baseline at 75% $\text{VO}_{2\text{max}}$.

Exercise at 75% $\text{VO}_{2\text{max}}$ resulted in significantly higher A Ca concentrations than exercise at 55% $\text{VO}_{2\text{max}}$ after 20 ($P \leq 0.05$), 25 ($P \leq 0.001$) and 30 min of exercise ($P \leq 0.001$) and after 25 min of recovery ($P \leq 0.01$).

Albumin

There was no main effect of *Intensity*, but there was a main effect of *Time* ($P \leq 0.001$) and an *Intensity* \times *Time* interaction ($P \leq 0.01$). Albumin concentrations increased with the onset of exercise and were higher than baseline between 7.5 min and the end of exercise at 65% $\text{VO}_{2\text{max}}$ (+4%; $P \leq 0.05$) and between 5 min of exercise and the end of exercise at 75% $\text{VO}_{2\text{max}}$ (+6%; $P \leq 0.05$) (Fig. 1D). Albumin concentrations peaked after 20 min of exercise and decreased thereafter, but remained higher than baseline until 5 min into recovery at 75% $\text{VO}_{2\text{max}}$ ($P \leq 0.001$). Albumin concentrations decreased below baseline 25 min into recovery and remained so until 90 min of recovery at 55% $\text{VO}_{2\text{max}}$ (-3 to -4%; $P \leq 0.01$). Concentrations decreased below baseline 20 min into recovery and remained so until 90 min of recovery at 65% $\text{VO}_{2\text{max}}$ (-3 to -5%; $P \leq 0.05$ to $P \leq 0.001$). Albumin concentrations did not decrease below baseline at 75% $\text{VO}_{2\text{max}}$.

Exercise at 75% $\text{VO}_{2\text{max}}$ resulted in significantly higher albumin concentrations than exercise at 55% $\text{VO}_{2\text{max}}$ after 25 min of exercise ($P \leq 0.05$).

Ca²⁺

There was no main effect of *Intensity*, but there was a main effect of *Time* ($P \leq 0.001$) and an *Intensity* \times *Time* interaction ($P \leq 0.001$). At 55% $\text{VO}_{2\text{max}}$, Ca^{2+} concentrations decreased after 10 min of exercise,

being significantly below baseline between 25 minutes and the end of exercise (Fig 2B) (-2%; $P \leq 0.001$). Ca^{2+} concentrations continued to decrease into recovery, remaining significantly below baseline until 90 minutes of recovery (-2 to -6%; $P \leq 0.001$). At 65% $\text{VO}_{2\text{max}}$ and 75% $\text{VO}_{2\text{max}}$ Ca^{2+} concentrations increased with the onset of exercise and were significantly higher than baseline between 2.5 and 10 min of exercise at 65% $\text{VO}_{2\text{max}}$ (+2 to +3%; $P \leq 0.001$) and between 2.5 and 7.5 min at 75% $\text{VO}_{2\text{max}}$ (+2 to +3%; $P \leq 0.001$). Thereafter, Ca^{2+} concentrations decreased and were significantly below baseline between 2.5 and 30 min of recovery at 65% $\text{VO}_{2\text{max}}$ (-3 to -4%; $P \leq 0.05$ to $P \leq 0.001$) and 75% $\text{VO}_{2\text{max}}$ (-3 to -4%; $P \leq 0.001$).

There were no significant differences between the three trials at any time point other than at baseline (Table 1), which created the significant *Intensity x Time* interaction.

Correlation Analyses

Changes in PTH were not correlated with changes in PO_4 or ACa in any trial. Across all data points PTH was significantly ($P \leq 0.001$) negatively correlated with Ca^{2+} at all intensities (Table 2).

Across PEAK data points, PO_4 was correlated with PTH at all exercise intensities ($r=0.661$ to 0.772) (Table 3) when the PTH series was lagged by 1 time point (3.5 min) behind the PO_4 series, suggesting that increases in PO_4 precede increases in PTH by 3.5 min. Ca^{2+} was most strongly correlated with PTH at all exercise intensities ($r=-0.902$ to -0.950) when there was no time lag, suggesting that increases in PTH occur within 3.5 min of a decrease in Ca^{2+} .

Across DEC data points, PO_4 , ACa and Ca^{2+} were correlated with PTH at all exercise intensities. PO_4 was most strongly correlated with PTH at all exercise intensities ($r=0.987$ to 0.995) (Table 3) when there was no time lag, suggesting that decreases in PTH occur within 8 min of a decrease in PO_4 .

Discussion

The novel findings from this study are: 1) changes in PTH, PO₄, ACa and Ca²⁺ occur within 2.5 min of the onset of exercise; 2) there is an initial decrease in PTH concentrations at the start of exercise that coincides with a significant increase in Ca²⁺ concentrations at the two higher exercise intensities; 3) peak PTH concentrations occur within 5–7.5 min of recovery; 4) increases in PO₄ precede increases in PTH; 5) decreases in Ca²⁺ precede increases in PTH; 6) post-exercise decreases in PTH concentrations are preceded by decreases in PO₄.

The pattern of change in PTH in this study is comparable to previous studies, with PTH concentrations increasing during exercise (15, 17–20) and peaking in the first minutes of recovery (12). The pattern of change in PTH was similar across the three exercise intensities, with an initial decrease from baseline to 5 min of exercise. We are the first to observe this initial response in PTH, due to the higher temporal frequency of blood sampling at the start of exercise compared with previous studies. This response requires verification from further studies and the use of even more frequent sampling. The lack of a resting control group in the present study means that we cannot confirm whether this is a characteristic physiological response to the onset of exercise or whether this reflects the circadian rhythm of PTH at the time of sampling. The nadir in PTH occurs between 08:00 and 10:00 (25–28) and our baseline blood was taken at 08:55, with exercise commencing at 09:02. If the initial decrease in PTH were due to the circadian rhythm, however, it would be expected that the decrease would have lasted longer than 5 min into exercise. Additionally, a decrease of 33% from baseline, followed by a rapid reversal in the direction of change, as shown here, has not been reported in circadian studies. Peak PTH concentrations have previously been shown to occur 15 min after exercise (12), due to a lower sampling frequency, but the results of the present study show that the peak in PTH after exercise occurs with 5 – 7.5 min of recovery (+73 to +110% from baseline). This peak is also transient; PTH concentrations start to decrease immediately after reaching peak concentrations. Transient spikes in PTH have been shown to be anabolic for bone (5), resulting in net bone gain (29). As such, our identification of peak PTH concentrations 5 – 7.5 min after exercise could be utilised as a tool for improving bone health amongst individuals at risk of fractures, stress

fractures or poor bone health, including the development of an exercise regime involving bouts of running sufficient to cause a spike in PTH concentrations, followed by rest periods to ensure that the spike is transitory. Further work is required to determine whether the response of PTH to this type of exercise is consistent and whether the magnitude of the changes in PTH are sufficient to induce such an effect.

Cross-correlations suggested that PTH secretion during exercise and recovery is controlled by a combination of changes in Ca^{2+} and PO_4 . Ca^{2+} is not routinely measured due to analytical difficulties; consequently ACa is estimated as a surrogate and has been shown clinically to be a reliable indicator of Ca metabolism at rest (30). We have shown different responses to exercise and recovery between ACa and Ca^{2+} and also different relationships with PTH; Ca^{2+} concentrations were correlated with PTH, whereas ACa was not. Albumin changes taking place during exercise will have a greater effect on the ACa estimation compared to the small effect that can occur on Ca^{2+} measurement; changes in pH were not sufficient to have a major effect on Ca^{2+} measurement by the blood gas analyser. The results support previous data (14, 15, 17–20) suggesting that changes in ACa do not explain the changes in PTH or regulation of PTH during exercise, because, as PTH is increasing, ACa either also increases (15, 17) or is unchanged (14, 18, 19). Scott *et al.* (19) argued that because both PTH and ACa were increased after 20 minutes of exercise, a decrease in Ca^{2+} could have occurred in the first few minutes of exercise, stimulating the secretion of PTH and causing serum Ca^{2+} concentrations to increase as a result of PTH-stimulated bone resorption and Ca^{2+} liberation. However, through frequent sampling, we have shown that ACa and Ca^{2+} , at 65% and 75% $\text{VO}_{2\text{max}}$, increase within 2.5 min of exercise, with ACa increasing and Ca^{2+} decreasing thereafter. Although it is well established that PTH responds rapidly to a reduction in Ca^{2+} at rest (1, 22), this is the first study to show that this rapid response also occurs during exercise. The lack of an initial increase in Ca^{2+} at 55% $\text{VO}_{2\text{max}}$ is surprising and the reason for this is currently unknown. The strong negative correlation of PTH and Ca^{2+} during exercise at all three intensities with a 0 time lag ($r=-0.902$ to -0.950) suggests that as Ca^{2+} decreases, PTH increases within 3.5 min. This negative cross-correlation

supports the findings of Bouassida *et al.* (11) who showed that as Ca^{2+} decreased during 42 minutes of running, PTH increased.

These findings suggest that Ca^{2+} may control PTH secretion during exercise. The reasons for the initial increase in Ca^{2+} at the start of exercise in the two higher exercise intensities are unknown, although this might be important in explaining the decreased PTH concentrations with the onset of exercise. It could have been related to exercise-induced acidosis occurring in the first few minutes of exercise, before aerobic metabolism stabilises (31, 32), which can increase Ca^{2+} concentrations (33) but have minimal effects on ACa. Blood pH did not, however, decrease significantly during exercise, suggesting that exercise-induced acidosis was not the reason for the initial increase in Ca^{2+} . Further mechanistic studies are needed to identify why this initial increase occurs, but it could be from calcium being released from other binding proteins such as transferrin (34) or calcium dissociating from PO_4 (35, 36).

Changes in systemic PO_4 can influence PTH secretion, with Ahmad *et al.* (37) showing that circadian changes in PO_4 precede changes in PTH. During the increase in PTH in the present study, PO_4 and PTH were most strongly positively cross-correlated at -1 time lag, suggesting that increases in PO_4 precede those in PTH by less than 3.5 min. This cross-correlation was not as strong, however, as the cross-correlation between Ca^{2+} and PTH, which might indicate that both PO_4 and Ca^{2+} are influential during the increase in PTH. Our data do not fully support that the exercise-induced increases in PTH are driven solely by increased PO_4 , as PO_4 increased with the onset of exercise despite the initial decrease in PTH. The increase in PO_4 might reflect release of PO_4 from PTH-induced bone resorption (15, 37, 38) towards the end of exercise, or that PO_4 is being released from muscle tissue, although this is speculative (39, 40). Taken together, these results suggest that Ca^{2+} is the stronger driver of PTH secretion and synthesis at the onset of exercise, however it is possible that the degree of association/dissociation between Ca^{2+} and PO_4 varies during exercise, meaning that PTH regulation might change accordingly.

With the decrease in PTH during recovery, the strongest positive cross-correlation between PO₄ and PTH occurred at a 0 time lag, suggesting that PTH decreased within 8 min of a decrease in PO₄. These findings support Scott *et al.* (15, 18–20), who showed that PO₄ followed the same response as PTH after exercise. If the decrease in PTH during recovery is explained by renal clearance (11), the strong cross-correlation may suggest that PO₄ is driving PTH clearance and over-riding Ca²⁺ regulation in recovery. Alternatively, the elevated PTH concentrations could be enhancing renal PO₄ excretion and causing a subsequent decrease in circulating PO₄ (41).

Reductions in vitamin D concentrations can contribute to an increase in PTH, as 1,25, dihydroxyvitamin D regulates the active transport of calcium and PO₄ absorption in the small intestine (42). Vitamin D status was not measured so we cannot confirm whether a change occurred during the study. The three trials were, however, completed within one month for each participant and the order of trials was randomised, meaning that, although changes in vitamin D concentrations could have occurred, they are unlikely to have influenced the results.

In conclusion, at the onset of exercise PTH transiently decreases then increases throughout exercise, peaking in the first minutes of recovery, before decreasing below the baseline concentration during ongoing recovery. Changes in Ca²⁺ and PO₄ occur in close temporal relation to changes in PTH. Cross-correlational analysis suggests that PTH secretion during exercise and recovery is controlled by a combination of changes in Ca²⁺ and PO₄ and that the mechanism might be different during exercise and recovery. A Ca may not be a suitable surrogate for Ca²⁺ when investigating the rapid response to exercise, since A Ca concentrations do not reflect temporal PTH responses or correlate strongly with PTH.

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Table Legends

Table 1. Baseline biochemistry across all trials.

Table 2. Pearson's correlation coefficient values for changes in PTH, with changes in PO₄, ACa and Ca²⁺.

Table 3. Maximum cross-correlation values and corresponding lag times for PTH with PO₄, ACa and Ca²⁺.

Figure Legends

Fig. 1. The percent change in baseline concentrations of PTH (A), PO₄ (B), ACa (C) and albumin (D) for all participants with 30 min of treadmill running at 55% VO_{2max} (open circles), 65% VO_{2max} (filled squares), 75% VO_{2max} (open triangles). Grey box denotes exercise. Data are mean±SD. ^a different ($P\leq 0.05$) from baseline (55% VO_{2max}) ^b different ($P\leq 0.05$) from baseline (65% VO_{2max}), ^c different ($P\leq 0.05$) from baseline (75% VO_{2max}). * 55% VO_{2max} different ($P\leq 0.05$) from 65% VO_{2max}, ^u 55% VO_{2max} different ($P\leq 0.05$) from 75% VO_{2max}, • 65% VO_{2max} different ($P\leq 0.05$) from 75% VO_{2max}.

Fig. 2. The percent change in baseline concentrations of PTH (A) and Ca²⁺ (B) for participants 5–10 with 30 min of treadmill running at 55% VO_{2max} (open circles), 65% VO_{2max} (filled squares), 75% VO_{2max} (open triangles). Grey box denotes exercise. Data are mean±SD. ^a different ($P\leq 0.05$) from baseline (55% VO_{2max}) ^b different ($P\leq 0.05$) from baseline (65% VO_{2max}), ^c different ($P\leq 0.05$) from baseline (75% VO_{2max}). * 55% VO_{2max} different ($P\leq 0.05$) from 65% VO_{2max}, ^u 55% VO_{2max} different ($P\leq 0.05$) from 75% VO_{2max}, • 65% VO_{2max} different ($P\leq 0.05$) from 75% VO_{2max}. Statistical analysis not reported or denoted for the PTH response in participants 5–10; data plotted for the comparison with Ca²⁺ only.

Table 1.

Measure	55% VO _{2max}	65% VO _{2max}	75% VO _{2max}
PTH (pmol·L ⁻¹)	2.62±0.88	2.51±0.50	2.63±0.60
PO ₄ (mmol·L ⁻¹)	1.14±0.12	1.17±0.25	1.12±0.16
ACa (mmol·L ⁻¹)	2.83±0.21	2.83±0.23	2.78±0.22
Albumin (g·dL ⁻¹)	4.60±0.14	4.63±0.19	4.57±0.22
Ca ²⁺ (mmol·L ⁻¹)	1.27±0.03 ^a	1.25±0.02	1.24±0.01

Data are mean±SD. ^a = Baseline Ca²⁺ at 55% VO_{2max} was significantly ($P\leq 0.05$) higher than at 65% and 75% VO_{2max}.

Table 2.

Exercise intensity	PO ₄	<i>r</i> value	
		ACa	Ca ²⁺
55% VO _{2max}	0.175	-0.160	-0.739 ^a
65% VO _{2max}	0.215	-0.077	-0.769 ^a
75% VO _{2max}	0.416	0.078	-0.790 ^a

^a = Significant correlation with PTH ($P \leq 0.001$).

Table 3.

Exercise intensity	PO ₄		ACa		Ca ²⁺	
	Time lag	<i>r</i> value	Time lag	<i>r</i> value	Time lag	<i>r</i> value
<i>PEAK data points (baseline to 5 min of recovery)</i>						
55% VO _{2max}	-1	0.661	0	-0.431	0	-0.902
65% VO _{2max}	-1	0.677	-2	0.550	0	-0.936
75% VO _{2max}	-1	0.772	-2	0.669	0	-0.950
<i>DEC data points (5 to 90 min of recovery)</i>						
55% VO _{2max}	0	0.995	0	0.761	+1	-0.794
65% VO _{2max}	0	0.987	0	0.908	0	-0.856
75% VO _{2max}	0	0.994	0	0.809	+1	-0.817

Fig. 2.

