The Effects of Treadmill Running on Bone Metabolism

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy by Jonathan Paul Richard Scott

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Disclosure

I hereby declare the present thesis has been composed by myself and that the work of which it is a record has been performed by myself except where assistance has been acknowledged. No part of this thesis has been submitted in any previous application for a higher degree. All sources of information have been specifically referenced.

Name: .....

Signature: .....

Date: .....

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Jonathan Paul Richard Scott

Abstract

This thesis examined the effects of an acute bout of endurance running on bone metabolism. Bone turnover markers in blood (β-CTX, P1NP, bone ALP, OC) and urine (free [f]PYD, fDPD), markers of calcium metabolism (PTH, albumin-adjusted calcium, phosphate [PO_4]), osteoprotegerin (OPG) and pro-inflammatory cytokines (TNF-α, IL-1β, IL-6), were measured during, and for up to four days post-exercise, in a series of laboratory studies.

Endurance running results in an increase in β-CTX concentrations, which can last for up to 4 days, but only if exercise is strenuous and exhaustive. The β-CTX response is only modestly affected by increasing cardiovascular exercise intensity and is of a greater duration with pre-exercise feeding compared with fasting. The timing of exercise, and the nutritional status of subjects prior to exercise, also affect both the direction and magnitude of the β-CTX response. Apart from transient increases during exercise, bone formation markers remain largely unchanged, suggesting that, at least up to 4 d post-exercise, acute, endurance running results in a transient imbalance in bone turnover, favouring bone resorption. Measured in fasted, early morning, second void samples, changes in creatinine (Cr)-corrected fPYD and fDPD, and fPYD and fDPD 'output', were not consistent with those in β-CTX. The two methods appear to be hampered by considerable individual variability in Cr concentrations and urine volume respectively.

Circulating OPG concentrations increase during endurance running, with the increase lasting for up to 24 h, but only if exercise is strenuous and exhaustive. This increase is not affected by training status, reduced recovery duration between two bouts of running, increasing cardiovascular exercise intensity or pre-exercise feeding. There was considerable variability in the magnitude of individual OPG responses and changes in OPG concentrations did not consistently reflect those in β-CTX.

The increase in PTH concentrations with endurance running was prompt and transient, being evident after 20 min, but returning to baseline within 30 min of recovery. This increase is not affected by improved training status, prior exercise or pre-exercise feeding, but occurs only above a certain cardiovascular exercise intensity. Wide variation in the PTH response to running suggests that the location of this threshold might vary between individuals. Changes in PTH were not associated with increases in bone formation markers and were more consistent with subsequent changes in β-CTX. Changes in systemic calcium and PO_4 are unlikely to mediate the increase in PTH during acute, endurance running.

Endurance running results in modest and transient increases in TNF-α and IL-1β, and marked increases in IL-6, the latter of which are enhanced with more strenuous exercise. The most marked and prolonged increase in IL-6 preceded the only sustained increase in β-CTX, suggesting a possible mediating role for circulating IL-6 in changes in bone resorption with endurance running.

Existing Publications

Part of the work contained in this thesis has been published and presented as follows:

Published Papers

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Abbreviations

$1,25(OH)_2D3$	1,25-dihydroxyvitamin D3
1CTP	c-terminal crosslinking telopeptides of type 1 collagen generated by metalloproteinase
25(OH)D	25-hydroxyvitamin D
5-HT	serotonin
5-HTT	5-HT transporter
ADOII	autosomal dominant osteopetrosis type II
AGHD	adult growth hormone deficiency
ACa	albumin-adjusted calcium
ALP	alkaline phosphatase
AN	anorexia nervosa
ARO	autosomal recessive osteopetrosis
AT	anaerobic threshold
ATPS	ambient temperature and pressure, saturated
AUC_I	area under the curve with respect to increase
B/I	bone/intestinal fraction of bone ALP
BMC	bone mineral content
BMD	bone mineral density
BMU	bone multicellular unit
BRC	bone remodelling compartment
BRU	bone remodelling unit
BTM	bone turnover markers
BSP	bone sialoprotein
BTPS	body temperature and pressure saturated
CK	creatine kinase
CT	calcitonin
CaSR	calcium-sensing receptor
CHO	carbohydrate
CIC-7	chloride channel 7
CLIA	chemoluminescence
CM	colorimetric
COX-2	cyclooxygenase-2
CTX	c-terminal crosslinking telopeptides of type I collagen
Cr	creatinine
DEXA	dual x-ray
DPD	deoxypyridinoline

ECG	electrocardiogram
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
ET	endurance-trained
EM	electrophoretic
FFM	fat-free mass
FMV	first morning void
GGHL	glycosyl-galactosyl-hydroxylysine
GHL	galactosyl-hydroxylysine
GnRH	gonadotropin-releasing hormone
GPI	hydrophobic glycosylphosphatidylinositol
GPI-PLC	GPI-specific phospholipase D GPI-PLC
GPI-PLD	GPI-specific phospholipase D GPI-PLD
HPLC	high performance liquid chromatography
HPT	hyperparathyroidism
HRR	heart rate reserve
Hyl	hydroxylysine
Hyp	hydroxyproline
iCa	ionised calcium
iv	intravenous
IL	interleukin
IL-1β	interleukin-1 beta
IL-6	interleukin-6
IL-6R	interleukin-6 receptor
IL-1ra	interleukin-1 receptor antagonist
IEE	intermittent, exhaustive exercise
IRMA	immunoradiometric assay
LMM	linear mixed model analysis of variance
LH	luteinising hormone
MLSS	maximum lactate steady state
MS	mass spectrometry
MSC	mesenchymal stem cells
MSX2	homeobox msh-like 2
MMP	martix metalloproteinases
NTX	n-terminal crosslinking telopeptides of type 1 collagen

OC	osteocalcin
OSX	osterix
OPG	osteoprotegerin
P1CP	c-terminal propeptides of type 1 procollagen
P1NP	n-terminal propeptides of type 1 procollagen
PECAM-1	platelet-endothelial cell adhesion molecule-1
PO_4	phosphate
PTH	parathyroid hormone
PTHrP	parathyroid hormone-related peptide
PYD	pyridinoline
RA	recreationally-active
RANK	receptor activator of nuclear factor (NF-kB)
RANKL	receptor activator of NF-kB ligand
RER	respiratory exhange ratio
RIA	radioimmunoassay
RPE	rating of perceived exertion
RUNX2	runt-related transcription factor-2
SDF-1	stromal cell-derived factor-1
sIL-6R	soluble interleukin-6 receptor
SNK	Student-Newman-Keuls
SMV	second morning void
TGF-β	tumour growth factor-beta
TNF-α	tumour necrosis factor-alpha
TRACP	tartrate-resistant acid phosphatise
$\mu\epsilon$	microstrain
VEGF	vascular endothelial growth factor-A
VO_{2max}	maximum rate of oxygen uptake
VT	Ventilatory threshold
W_{max}	work load max

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CHAPTER I

INTRODUCTION

Bone's inanimate appearance belies a flexible, highly dynamic tissue that is constantly in flux, fulfilling a range of critical roles within the human body. Beyond its more obvious roles including the protection of internal organs and supporting movement by providing a light but strong scaffold with attachment points for musculotendinous units, bone also acts as both a sink and reservoir for calcium, an ion essential for cellular metabolism and function, while the marrow cavity provides an essential source of hematopoietic, mesenchymal and endothelial stem cells. There is also emerging evidence that bone may also act as an endocrine organ, key in the regulation of energy metabolism (Fukumoto and Martin, 2009). To act as both an appropriate scaffold and a reservoir, bone must be capable of adapting to the predominant biomechanical challenges placed upon it whilst simultaneously making components of its structure accessible in response to subtle changes in the systemic environment.

Bone achieves some of these discrete tasks through the remarkable process of bone remodelling or bone turnover, in which existing bone is removed (bone resorption) and new bone is formed in its place. The resorption and formation processes are dependent on the orchestrated actions of osteoclasts and osteoblasts that are temporally and spatially coupled, with a positive balance during growth and a negative balance with ageing, with the latter leading to osteoporosis. With the exception of these scenarios, however, the tight coupling between resorption and formation ensures that there is no loss of skeletal integrity and that the skeleton is a readily accessible source of calcium and phosphate (PO_4) while remaining optimally functionally adapted.

1.1 The importance of physical activity in bone health

Given the necessity to adapt to the biomechanical demands placed upon it, in addition to responding to hormones (Tam *et al.*, 1982), growth factors (Fox and Lovibond, 2005) and cytokines (Kwan Tat *et al.*, 2004), it is unsurprising that bone is highly sensitive to external mechanical loads and remains responsive to loading throughout the human lifespan. A high level of physical activity is associated with higher bone mineral density (BMD) during childhood and is an important factor in the achievement of peak bone mass (Valdimarsson *et al.*, 1999; Nordstrom *et al.*, 2005; Valdimarsson *et al.*, 2005). Cross-sectional, observational studies of unilateral physical activities such as tennis, clearly demonstrate the responsiveness of the child and adolescent skeleton to physical loading during growth (Kannus *et al.*, 1994; Kannus *et al.*, 1995; Haapasalo *et al.*, 1996). These findings are confirmed by controlled, prospective exercise intervention trials that show improvements in both BMD and bone size (Linden *et al.*, 2006), both of which independently influence bone strength and resistance to fracture (Cummings *et al.*, 1995; Duan *et al.*, 2001; Seeman *et al.*, 2001).

Importantly, high levels of physical activity in the early years might also reduce fracture risk in older age (Nordström *et al.*, 2005).

Although high quality, randomised, controlled trials remain relatively rare, there is considerable evidence that exercise and physical activity can also have positive effects on bone in elderly populations (Wolff *et al.*, 1999) with aerobic and resistance exercise increasing BMD at the spine (Bonaiuti *et al.*, 2002; Martyn-St James and Carroll, 2006) while walking exercise may be effective at the hip (Bonaiuti *et al.*, 2002; Martyn-St James and Carroll, 2008). Importantly, physical activity is also associated with a reduced risk of fracture and, in moderately-active populations, a reduced risk of falling (Moayyeri, 2008). Whether the several percent increase in BMD that is reported with exercise is of biological significance – and thus contributes to the reduction in fracture risk – remains to be fully elucidated (Moayyeri, 2008). The lack of a strong association between changes in BMD and fracture risk in these populations might be related to other effects of mechanical loading such as increases in cross-sectional area, (Leppänen *et al.*, 2008), bone mineral content (Leppänen *et al.*, 2008) and trabecular connectivity density (Issever *et al.*, 2003) that may also contribute to fracture resistance.

1.2 Detrimental effects of physical activity on bone health

Despite this positive picture, there are an increasing number of reports of negative associations between exercise and bone health including a reduction in BMD at certain anatomical sites in some endurance athletes and stress fractures (SFx) in both athletes and military recruits. As would be anticipated from the generalised beneficial effect of exercise on bone health, cross-sectional studies of athletic populations, particularly those involved in weight-bearing exercise, report increased BMD at sites subject to strain during exercise compared to non-athletic controls (Brahm *et al.*, 1997c; Stewart and Hannan, 2000; Kemmler *et al.*, 2005; Fredericson *et al.*, 2007). However, also consistent with the beneficial effects of mechanical loading, in sports associated with long periods of negligible loading such as road cycling, this positive relationship has not been observed and can actually result in reduced BMD (Sabo *et al.*, 1996; Stewart and Hannan, 2000; Nichols *et al.*, 2003). Despite observing increased BMD at high strain sites in runners, some studies did not observe this effect at all anatomical sites and an increasing number of studies are reporting reduced BMD at the lumbar spine, a site not subjected to the strain levels seen with running in the upper and lower legs (Blinan *et al.*, 1989; Hetland *et al.*, 1993a; Hetland *et al.*, 1993b; Hetland *et al.*, 1994; Hind *et al.*, 2006).

A SFx is a partial or complete bone fracture resulting from its inability to withstand the repeated application of a sub threshold stress (McBryde, 1985; Martin and McCulloch, 1987). Although rare in the general population, they are commonly reported injuries in military recruits (Jones *et al.*, 1993; Beck *et al.*, 2000; Shaffer *et al.*, 2005), athletes, particularly runners (Goldberg and Pecora, 1994; Johnson *et al.*, 1994) and dancers (Frusztajer, 1990). First described in 1855 in Prussian soldiers

(Breithaupt, 1855) and later confirmed clinically by Stechow (1897), SFx are commonly associated with a new, strenuous and repetitive activity that results in the deformation of bone by muscular force, muscle fatigue or mechanical load. Deformation is presumed to lead to microdamage and, if left unchecked, with further loading of a similar nature, this microdamage progresses into a SFx, although the exact mechanism remains unclear.

In military recruits, SFx injury during basic training carries a high cost to national defence forces due to lost training time, medical care, and recruit attrition. In financial terms alone, the burden is considerable: it has been estimated that in a one year period, from one US Army training base, $26 million was lost in training costs for the 749 soldiers who were discharged from training with SFx, at an average of more than $34,000 per soldier (USARIEM, 2006). In the UK, when the cost of working days lost, wastage through medical discharge, and medical support Service-wide for all Arms is considered, the cost of stress fractures per annum is estimated to exceed £10 million (SO1, Consultant in Occupational Medicine, UK Army Training and Recruiting Agency). In the last decade, a concerted, collaborative effort between the US Army, Navy, the Israeli Defence Forces, and researchers from the US and Israel have successfully reduced the incidence of SFx by more than 50% through a combination of empirical training-based interventions including reducing the overall mileage covered, changing footwear, orthoses and eliminating formation marching (Friedl *et al.*, 2008). Despite this reduction, SFx remains a significant problem, with a recent study of the US Marine Corps Recruit Depot reporting a 6.6% incidence of SFx in female recruits, equivalent to 1.0 per 1000 training days of exposure (Rauh *et al.*, 2008).

1.3 Bone turnover markers and the role of bone turnover in the detrimental effects of physical activity on bone health

The isolation and characterisation of cellular and extracellular parts of the skeletal matrix have allowed the development of markers that are considered to reflect specific aspects of the bone turnover process, either bone resorption or bone formation. Currently available bone turnover markers (BTM) are typically classified according to the metabolic process they reflect and include both enzymes and non-enzymatic peptides derived from cellular and non-cellular compartments of bone. Most markers of bone resorption are related to collagen breakdown products but others include non-collagenous matrix proteins such as bone or osteoclast-specific enzymes. Markers of bone formation are either by-products of collagen neosynthesis or osteoblast-related proteins. Compared to previous techniques such as bone biopsies, these BTM are less-invasive, inexpensive and can be measured repeatedly. When measured carefully and interpreted correctly, BTM have provided an improved clinical understanding of the relationship between bone turnover, BMD and bone fragility and are an additional tool in the diagnostic and therapeutic assessment of metabolic bone disease (Szulc and Delmas, 2008).

Changes in bone turnover have been implicated in both the negative effects of chronic exercise on BMD and in the pathophysiology of SFx and BTM have provided novel insights into their individual pathologies. Much of the focus of research into the detrimental effects of chronic exercise training on BMD has focused on female athletes. Initially it appeared that estrogen deficiency explained the negative changes in BMD with chronic exercise (Drinkwater *et al.*, 1984; Myerson *et al.*, 1992; Myburgh *et al.*, 1993; Rencken *et al.*, 1996; Gremion *et al.*, 2001). Recent work, however, suggests that male and female endurance runners are equally prone to reduced BMD at the lumbar spine (Hind *et al.*, 2006) although, unlike in females, low BMD in men appears to be unrelated to levels of gonadotropins, estrodial or testosterone (MacDougall *et al.*, 1992; Hetland *et al.*, 1993; Bennell *et al.*, 1996). More recently, estrogen deficiency has been shown to occur secondary to an energy deficit in women (Williams *et al.*, 2001). Taken together, these findings suggest that the detrimental effects of strenuous exercise on bone health are neither gender specific nor solely related to changes in hormonal status, but may occur in any athlete with an imbalance between energy intake and expenditure.

The outcome of an energy imbalance is a decrease in bone formation markers with no change in bone resorption markers (Zanker and Swaine, 2000) although, in conditions of severe energy restriction, in addition to a decrease in bone formation, an increase in bone resorption also occurs (Ihle and Loucks, 2004) resulting in a remodelling balance that favours bone resorption. These changes are, at least in part, likely to underlie the decreased BMD reported in some endurance athletes (Zanker and Swaine, 1998; Zanker and Swaine, 2000; Zanker and Cooke, 2004). There is also some evidence that a single bout of exercise might result in a remodelling balance, at least in the short term, that also favours bone resorption, with exercise increasing bone resorption but not formation (Guillemant *et al.*, 2004), thus providing a secondary mechanism by which strenuous training might, over time, negatively affect BMD.

Two theories currently exist to explain how mechanical loading can lead to SFx. The first theory contends that SFx result directly from the development, accumulation and growth of microcracks (Evans and Riolo, 1970; Carter and Hayes, 1977; Carter *et al.*, 1981a; Carter *et al.*, 1981b; Carter and Caler, 1985; Caler and Carter, 1990; Nunamaker *et al.*, 1990) and are purely a result of mechanical damage (Figure 1.1). This 'mechanical' theory of SFx development is not, however, supported by data from *ex-vivo* experiments using prepared bone specimens (Schaffler *et al.*, 1989) or epidemiological data from populations with a high rate of SFx (Milgrom *et al.*, 1985). When cortical bone specimens are subjected to uniaxial strains within the physiological range [< 2000 microstrains ($\mu\epsilon$)] (Burr *et al.*, 1996; Hoshaw *et al.*, 1997; Fyhrie *et al.*, 1998), bone can sustain tens of millions of cycles without fracturing (Schaffler *et al.*, 1989). Assuming one cycle is equivalent to one, 1.5 metre stride, 10 million cycles would represent 1500 km of locomotion, which is four to five times the distance estimated to be covered by military recruits during 12 weeks of basic training (Jones *et al.*, 1989). In addition, studies of recruits undergoing military training report the peak incidence of SFx

occurrence to be between three and six weeks, and a relatively small number of recruits suffer from stress fractures despite all recruits undergoing a similar volume of loading. These findings suggest that mechanical damage alone does not appear to be sufficient to account for the time course of SFx development *in vivo* and that other factors may be involved.

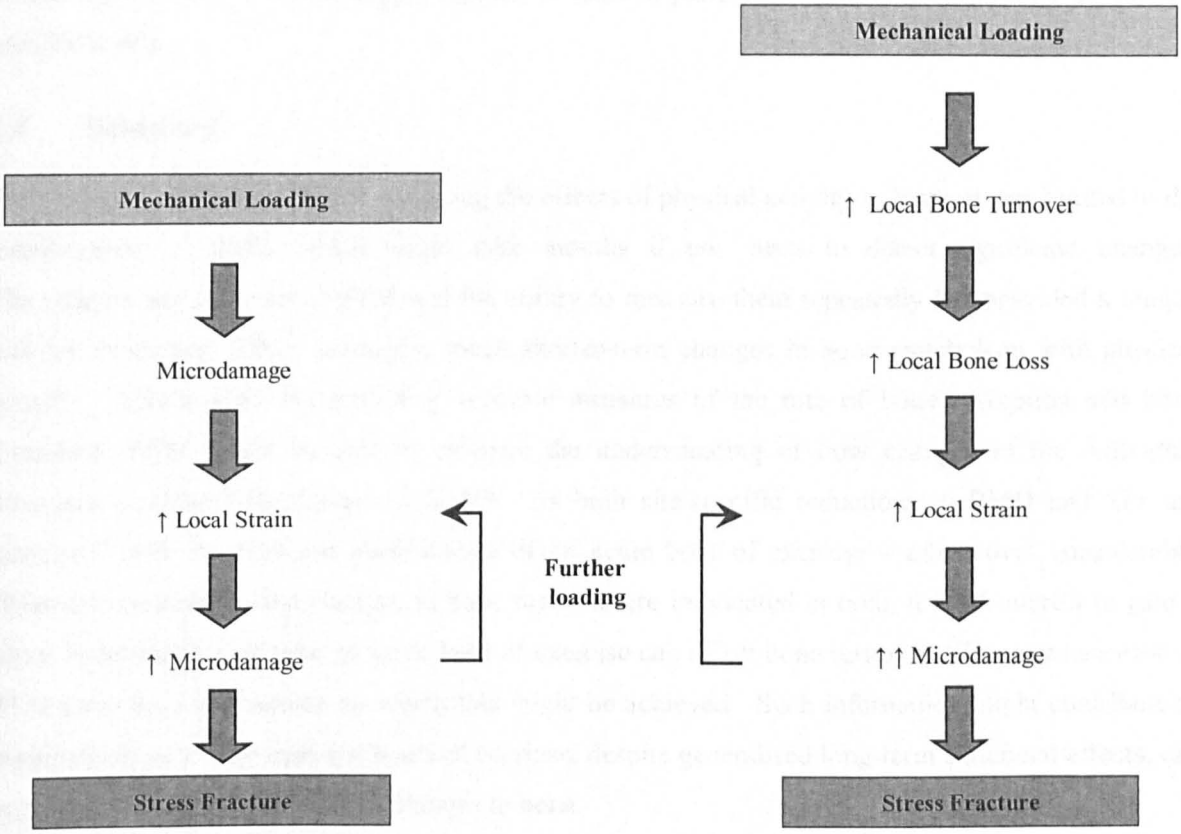

Figure 1.1. The 'mechanical' and 'metabolic' theories of stress fracture injury (SFx). In the mechanical theory (left hand side), damage is a direct result of loading only, with damage leading to increased local strain during subsequent loading, more damage, and then SFx. In the metabolic theory (right hand side), the increased local strain occurs not only from damage but also from a localised osteoporosis resulting from a targeted bone remodelling response to loading (and damage). During subsequent loading, further damage occurs – which may also stimulate a further remodelling response – leading to SFx.

An alternative, and now more widely accepted theory, holds that mechanical loading can directly result in the stimulation of targeted bone remodelling without the presence of microdamage, modelling SFx as a positive feedback mechanism. In this 'metabolic' model, the direct stimulation of targeted bone remodelling results in localised increases in bone resorption leading to increased porosity and reduced bone mass (Figure 1.1). A reduction in bone mass in these relatively osteoporotic areas will in turn affect important mechanical properties such as stiffness, and continued loading results in marked increases in stresses and strains, microdamage, further remodelling and eventually SFx (Johnson *et al.*, 1963; Schaffler *et al.*, 1989; Schaffler *et al.*, 1990; Martin, 1995). Thus, this model does not rule out the possibility that loading directly results in damage, but suggests that bone

remodelling, responding to both loading itself and to the damage induced by loading, may accelerate damage accumulation and, as a result, is an important contributory factor to SFx development. Military training has been shown to increase bone turnover as evidenced by increases in both bone resorption and bone formation markers after only four weeks (Evans *et al.*, 2008). This overall acceleration in bone turnover might explain, at least in part, the increased susceptibility of military recruits to SFx.

1.4 Summary

Prior to the availability of BTM, assessing the effects of physical activity in humans was limited to the measurement of BMD which might take months if not years to detect significant changes. The relative convenience of BTM and the ability to measure them repeatedly has provided a unique tool for examining, albeit indirectly, much shorter-term changes in bone metabolism with physical activity. Additionally, by providing separate measures of the rate of bone resorption and bone formation, BTM might be able to enhance the understanding of how changes in the individual processes contribute to changes in BMD. As both site-specific reductions in BMD and SFx are associated with the repeated performance of an acute bout of exercise – albeit over considerably different timescales – and changes in bone turnover are implicated in both, it is of interest to gain a better understanding of how an acute bout of exercise can affect bone turnover. The measurement of BTM provides a mechanism by which this might be achieved. Such information might contribute to explanations as to why repeated bouts of exercise, despite generalised long-term beneficial effects, can occasionally result in detrimental changes to bone.

The main aim of this thesis was to examine changes in bone turnover with acute exercise in order to contribute to the understanding of how the bone turnover process responds to an acute bout of weight-bearing exercise. This is presented in the following nine chapters:

- The review of literature (Chapter II) presents a conceptual background for the studies that follow. A brief history of BTM is presented as is an examination of pre-analytical factors that may affect their measurement and thus their interpretation. A chronological history of studies of acute exercise and BTM is presented followed by a discussion of the possible role of parathyroid hormone (PTH) in modulating the bone turnover response to exercise and the mechanisms by which PTH secretion with exercise might be altered;
- In the General Methodology (Chapter III) the procedures followed, the equipment used and the methods of data collection and analysis are presented;
- In Study I (Chapter IV), the aim was to examine the effects of training status on the metabolic response of bone to an acute bout of exhaustive, treadmill running;

- In Study II (Chapter V), the aim was to examine the effects of reducing the recovery period between two acute bouts of treadmill running from 23 h to 3 h on the metabolic response of bone;
- In Study III (Chapter VI), the aim was to examine the effect of exercise intensity on the metabolic response of bone to an acute bout of treadmill running;
- In Study IV (Chapter VII), the aim was examine the effects of acute feeding on the metabolic response of bone to a subsequent bout of treadmill running;
- The aim of Chapter VIII was to explore a novel hypothesis that increases in circulating concentrations of the pro-inflammatory cytokines tumour necrosis factor (TNF)-α, interleukin(IL)-1β and IL-6 might be mediating changes in bone turnover during acute running;
- In the final chapter (Chapter IX) the findings of each study are discussed in relation to each other and to existing knowledge in this area. Possible mechanisms by which changes in bone turnover in response to acute exercise result in the responses observed during periods of repeated exercise, such as military training and athletic training, are discussed.

CHAPTER II

REVIEW OF LITERATURE

2.1 Bone remodelling

2.1.1 Bone remodelling: a five stage cycle

Bone remodelling is achieved through the coordinated activity of a group of cells termed a bone remodelling unit (BRU) containing cells responsible for bone resorption known as osteoclasts and those responsible for bone formation known as osteoblasts. Together these cells coordinate a bone remodelling cycle that starts and ends with a period of quiescence and consists of four distinct phases: activation, resorption, reversal and formation.

2.1.1.1 Quiescence

Unlike young growing animals, where almost all free bone surfaces show evidence of remodelling activity, in humans and other adult, large animals ~80% of the trabecular and ~95% of the intracortical bone surface is inactive in this regard. At this time, the bone surface is covered by a layer of thin bone lining cells (Miller *et al.*, 1980) that arise from terminal transformation of osteoblasts (see *bone formation*) (Figure 2.1). From these cells extend processes that join with similar processes from osteocytes (also from the osteoblast lineage) that are entombed in the bone matrix and connected via channels known as canaliculae.

2.1.1.2 Activation

Activation refers to an initiating event that changes a quiescent bone surface into a remodelling one and involves the recruitment of mononucleated osteoclast precursors from cells in the monocyte-macrophage lineage in the circulation (Roodman, 1999). The magnitude and precise location of the subsequent remodelling response is thought to be signalled by osteocytes to lining cells (Parfitt, 2001) which in turn transmit this information to the marrow environment, one of the sources of precursor cells (Hauge *et al.*, 2001; Parfitt, 2001). Prior to the resorption process, lining cells produce collagenase, which digests the layer of unmineralised matrix exposing the bone surface (Chambers *et al.*, 1985). However, as lining cells still cover the bone surface and osteoclasts arise from precursors in the adjacent hematopoietic marrow, despite exposure of the matrix beneath, no pathway to it exists. Consistent with Rasmussen and Bordier's idea of a canopy (Rasmussen and Bordier, 1974), Hauge *et al.* (2001) showed a layer of lining cells persists during remodelling, creating a bone remodelling compartment (BRC), a finding recently confirmed by Andersen *et al.* (2009). Importantly, Hauge *et al.* (2001) also showed that although this layer of cells persists during remodelling, it is released from the surface by a disruption of junctions between the lining cells and

entombed osteocytes (Figure 2.1). This retraction provides the pathway by which osteoclast precursors gain access to the exposed matrix in the BRC and the process of resorption can commence.

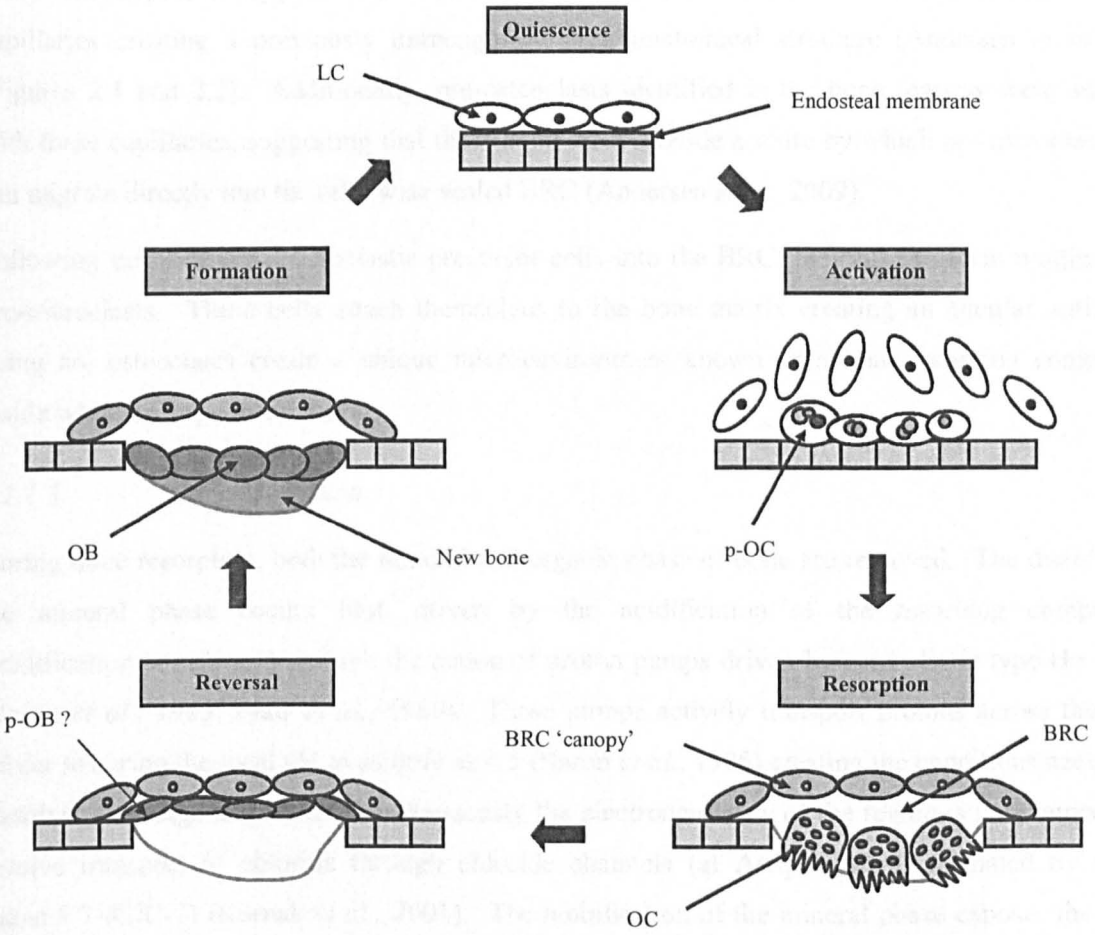

Figure 2.1. The normal 5-stage remodelling sequence in adult bone starting and ending with quiescence. The identification of a layer of cells covering the remodelling site creating a bone remodelling compartment (BRC) (Hauge *et al.*, 2001) and the subsequent identification of capillaries associated with the cells of this layer (Andersen *et al.*, 2009) provides a microanatomical arrangements that contributes the to control bone resorption and formation at remodelling sites. LC, lining cell; p-OC, pre-osteoclast; OC, mature, bone resorbing osteoclast; p-OB, pre-osteoclast; OB, mature, bone-forming osteoblast.

The signals that initiate retraction are yet to be precisely determined. PTH is known induce an elongation of osteoblasts and an increase in intercellular gaps (Jones and Boyde, 1976a; Jones and Boyde, 1976b; Jones and Ness, 1977) while tumour growth factor (TGF)-β induces both elongation and retraction through a matrix metalloproteinase (MMP)-dependent pathway (Karsdal *et al.*, 2003). Perez-Amodio *et al.* (2004) showed that on contact with both peripheral blood mononuclear cells and osteoclasts, osteoblasts were activated resulting in their retraction. In this study, both tumour necrosis factor (TNF)-α and TGF-β induced elongation but not retraction of osteoblasts while MMP inhibition completely blocked retraction. The physical contact between cell types suggests the possibility of a

cell-cell interaction and a role for adhesion molecules, although the identification of these molecules remains unknown.

More recently, the canopy cells that form the roof of the BRC have been observed in association with capillaries creating a previously unrecognized microanatomical structure (Andersen *et al.*, 2009) (Figures 2.1 and 2.2). Additionally, pre-osteoclasts identified in the bone marrow were associated with these capillaries, suggesting that these capillaries provide a route by which pre-osteoclastic cells can migrate directly into the otherwise sealed BRC (Andersen *et al.*, 2009).

Following infiltration of osteoclastic precursor cells into the BRC, they fuse to form multinucleated pre-osteoclasts. These cells attach themselves to the bone matrix creating an annular seal, and in doing so, osteoclasts create a unique microenvironment known as a bone-resorbing compartment inside which resorption can occur.

2.1.1.3 Bone resorption

During bone resorption, both the mineral and organic phase of bone are removed. The dissolution of the mineral phase occurs first, driven by the acidification of the resorbing compartment. Acidification is achieved through the action of proton pumps driven by osteoclastic type H+ ATPase (Baron *et al.*, 1985; Blair *et al.*, 1989). These pumps actively transport protons across the ruffled border lowering the local pH to as little as 4.5 (Baron *et al.*, 1985) creating the conditions necessary to dissolve the inorganic phase. Simultaneously the electroneutrality of the region is maintained by the passive transport of chloride through chloride channels (al Awqati, 1995) mediated by chloride channel 7 (CIC-7) (Kornak *et al.*, 2001). The mobilisation of the mineral phase exposes the organic phase, the dissolution of which is mediated through the action of Cathepsin K, that degrades type 1 collagen (Gowen *et al.*, 1999; Saftig *et al.*, 2000; Lark *et al.*, 2002; Garnero *et al.*, 2003b; Fratz-Zelman *et al.*, 2004) although recent evidence indicates that, in addition to its primary role in the dissolution of the inorganic matrix, acidification by the V-ATPase and ClC-7 plays a secondary role in the degradation of the organic matrix (Henriksen *et al.*, 2006).

The role of matrix metalloproteinases in bone resorption is less clear. Reports that bone resorption is attenuated in mice carrying a mutation in the site in type 1 collagen that is targeted by neutral collagenases (Zhao *et al.*, 1999), suggest a role for the participation of these enzymes in the resorptive process. Where cathepsin K mediated bone resorption is deficient, such as in pycnodysostosis (Nishi *et al.*, 1999) and cathepsin K deficient mice (Kiviranta *et al.*, 2005), there is evidence of enhanced MMP activity indicating a compensatory response by these proteases. In conditions of defective cathepsin K function, it is also possible that, in addition to bone lining cells (Everts *et al.*, 2002), MMPs also participate in removal of the undigested matrix left by the osteoclast (Henriksen *et al.*, 2006). Some MMPs (*e.g.* MMP-13) are also amongst the key enzymes governing osteoclast movement and the initiation of bone resorption (Delaisse *et al.*, 2003).

The products of bone degradation are endocytosed by the osteoclast and transported to and released at the cell's antiresorptive surface (Nesbitt and Horton, 1997). The removal of the matrix results in the creation of cavities known as Howship's lacunae. These lacunae exist on the surface of cancellous bone and in cylindrical tunnels in the cortex of cortical bone. In trabecular bone at least, only about two-thirds of the final depth of the cavity, can be accounted for by multinucleated osteoclast resorption (Eriksen *et al.*, 1986). The remainder is thought to be eroded much more slowly by mononuclear cells which could be potential precursor cells that have failed to fuse, or resorbing monocytes (Dominguez and Mundy, 1980; Dempster *et al.*, 2005). In addition, these monocytes may also stimulate osteoclasts by release of prostaglandins (Dominguez and Mundy, 1980).

It is possible that osteoclasts may go through several resorption cycles before their activity ceases (Kanehisa *et al.*, 1990) but, once resorption ends, they either undergo fission back to mononuclear cells or die as a result of apoptosis (Väänänen, 2005). In the case of multinucleated osteoclasts, there is no evidence that they will fission but are removed solely by apoptosis after a lifespan of approximately 12 d. This lifespan, governed by the timing of apoptosis, is crucial as it dictates the volume of bone removed in the resorption phase. The factors that govern apoptosis remain to be fully described. Both *in vitro* and *in vivo*, TGF-β promotes osteoclast apoptosis (Hughes *et al.*, 1996), exerting its effect *in vivo* by acting on osteoblasts (Fox and Lovibond, 2005). As the depth of resorption cavities is consistent in trabecular and cortical bone and, as the cavity grows, the proximity of the osteoclast to osteocytes decreases, it has also been proposed that the apoptosis could also be controlled by the bone lining cell-osteocyte network (Parfitt, 1984).

2.1.1.4 Reversal - the coupling of bone formation to bone resorption

Histologically, the reversal phase is characterised by a Howship's lacuna lacking osteoclasts. During the reversal phase, the resorption cavity is populated by several types of mononucleated cells including monocytes and osteocytes that have been liberated from the matrix during resorption. These cells deposit a thin layer of highly mineralized, collagen-poor bone matrix known as cement substance. Most important of these cells are the pre-osteoblasts that have been recruited to the region to initiate the formation phase of the cycle (Baron *et al.*, 1980). The tight coupling of bone formation to bone resorption is the most important feature of remodelling. Without effective coupling, an imbalance between the two processes would exist resulting in either a net gain or loss of bone with each remodelling cycle potentially leading to osteopetrosis or osteoporosis respectively. It is likely that the key coupling signals that initiate the recruitment of osteoblasts to the resorption cavity are sent during the reversal phase. Although yet to be fully defined, there are several hypotheses regarding the nature of these signals.

One theory implicates changes in local strain as the coupling signal (Smit and Burger, 2000; Smit *et al.*, 2002). Using three dimensional finite element models of both an osteonic tunnel and a Howship's lacuna, Smit and Burger, (2000) determined the strain fields within both cortical and trabecular bone during the remodelling process. In cortical bone, the strain field intensity was reduced by 70% in front of the tunnel excavated by osteoclasts. This area corresponds well with the location of active osteoclasts resorbing further bone. In contrast, behind the tunnel, or cutting cone, strain increases by 40% with the location corresponding well with the area where osteoclasts are inhibited and osteoblasts are activated. In the model of trabecular remodelling, strains were increased at the base of the lacuna and increased with lacuna depth. At 60um strain rises by 100% while at the lacuna border, strain was decreased in the direction of loading and increased perpendicular to it. This showed that the pattern of strain fields during remodelling was essentially the same in cortical and trabecular bone, with reduced strain in the direction of loading and increased strain perpendicular to it, and that osteoclastic activity is associated with reduced strain and osteoblastic activity with increased strain. It is proposed that this gradient results in the sequential activation of osteoclasts and osteoblasts, and bone multicellular unit (BMU) coupling may be a strain-relegated phenomenon (Smit and Burger, 2000).

Further finite model analysis has implicated strain-induced fluid flow as a possible regulator of both BMU coupling and osteonal alignment. Under loading, Smit *et al.* (2002) showed that at the tip of the cutting cone, fluid flows into the bone matrix from the resorption space, while towards the rear of the tunnel – the region of the reversal zone and closing cone - fluid is pressed out of the bone matrix and into the resorption space. Their model also demonstrated volumetric expansion of the bone tissue at the site of fluid influx into the tissue, while volumetric compression occurred at the site of fluid efflux. At the cutting cone, where expansion occurred, the amplitude of fluid flow was one sixth that of the flow at the closing cone while and at a depth of 10 um the direction of fluid flow at the cutting cone was reversed and that fluid stasis will occur. Smit *et al.* (2002) propose that the much reduced fluid flow at the cutting cone leads to a lack of stimuli to local osteocytes that the continued recruitment of osteoclasts to the area. In contrast, the increased fluid flow to the other areas of the osteonic tunnel serves to activate osteocytes to recruit osteoblasts and so local fluid flow patterns, in response to loading, provide an explanation not only for the coordinated actions of osteoblasts and osteoclasts but also for the progression of a BRU and the alignment of secondary bone with the direction of the dominant loading.

A second theory suggests that osteoclastic bone resorption may release growth factors present in the matrix that act as chemo-attractants for osteoblast precursors and stimulate osteoblast proliferation and differentiation. This hypothesis would explain the correct spatial localisation of the osteoblasts and the recruitment of the appropriate number of osteoblasts to replace the volume of bone removed during resorption, which in turn will determine the quantity of growth factor released. TGF-β is of particular interest as it prolongs osteoblast lifespan by inhibiting apoptosis and correlates with

histomorphometric indices of resorption, formation, and serum levels of bone formation markers (Pfeilschifter *et al.*, 1998). TGF-β activity is evident in the medium harvested from bone resorbing cells and is lessened with calcitonin inhibition of resorption suggesting that its appearance in the medium is a result of bone resorption (Pfeilschifter and Mundy, 1987). TGF-β is also a chemoattractant for osteoblast-like cells (Pfeilschifter *et al.*, 1990). As TGF-β is stored in the matrix, complexed to a protein that appears to function as a structural matrix protein (Dallas *et al.*, 1995), and osteoblast-like cells express different latent forms, possibly reflecting their state of maturation (Dallas *et al.*, 1994), these findings provides a mechanism by which TGF-β might be able to co-ordinate formation bone formation following its release from the matrix by bone resorption (Bonewald and Dallas, 1994; Dallas *et al.*, 1994; Dallas *et al.*, 1995).

Rather than factors released by osteoclast activity, a third possible mechanism involves the osteoclast itself. The conditions autosomal recessive osteopetrosis (ARO) and autosomal dominant osteopetrosis type II (ADOII) are associated with reduced bone resorption but either unaltered or increased bone formation (Bollerslev *et al.*, 1989; de Vernejoul and Bénichou, 2001; Alatalo *et al.*, 2004; Del Fattore *et al.*, 2005) and an increased number of non-resorbing osteoclasts that are otherwise normal in appearance (Bollerslev *et al.*, 1993). In the case of ADOII, the condition appears to be caused by a mutation that affects the function of CIC-7 (Del Fattore *et al.*, 2008) whereas recessive mutations or compound hetrozygozity within the TC1RG1 gene – which is associated with the function of the a3 subunit of the osteoclast proton pump (a3-V-ATPase) – account for about 50% of ARO cases (Segovia-Silvestre *et al.*, 2009). In contrast, Pycnodysostosis, a condition affecting the gene that encodes cathepsin K, presents with a considerably different phenotype and is associated with reduced resorption (Nishi *et al.*, 1999) but a normal number of osteoclasts (de Vernejoul and Bénichou, 2001). Despite the presence of normal numbers of osteoclasts, in these patients, there is no apparent uncoupling of formation and resorption, and they characteristically have poorly remodelled bone (Sarnsethsiri *et al.*, 1971; Fratzl-Zelman *et al.*, 2004). These mutations interfere with the ability of the osteoclast to acidify the resorption lacunae and as a consequence the normal degradation of the matrix. *In vitro*, proton pump (baflinomycin) and CIC-7 inhibition increases osteoclast survival and number, but attenuates individual osteoclast acidification capacity – assessed from c-terminal cross-linking telopeptides of type 1 collagen (CTX) production – and produces an increased number of non-resorbing osteoclasts. In contrast, inhibition of cathepsin K results in no increase in osteoclast number but a complete lack of resorption activity as measured by CTX (Karsdal *et al.*, 2005).

Taken together, these studies that show normal or increased bone formation despite defective osteoclast function (*e.g.* in ADOII) and the apparent importance of osteoclast number rather than resorption activity (Alatalo *et al.*, 2004), suggest that coupling might result from a factor secreted directly by osteoclasts rather than by bone resorption itself (Karsdal *et al.*, 2007). Subsequently, Karsdal *et al.* (2008) showed that conditioned medium from osteoclasts cultured either

on bone (resorbing osteoclasts), or plastic (non-resorbing osteoclasts) results in bone formation when added to osteoblast cultures. The osteoclast as the source of the anabolic signal was confirmed when only the conditioned media from the osteoclasts induced bone formation, whereas no effect was observed using the non-conditioned medium (Karsdal *et al.*, 2008). Such a mechanism might explain why intermittent PTH has no anabolic effects in c-*fos*−/− animals (Demiralp *et al.*, 2002), which have no osteoclasts but whose osteoblasts *in vitro* apparently function normally, and an attenuated effect following OPG treatment (that decreases osteoclast number) (Koh *et al.*, 2005), but is preserved in normal in c-src−/− mice (Koh *et al.*, 2005), which have increased numbers of non-resorbing osteoclasts. The identity of such a factor is yet to be identified, but several possibilities have been suggested including TGF-B, insulin growth factors, bone morphogenetic proteins, EphrinB2, EphB4, TRACP and cardiotrophin-1 (Karsdal *et al.*, 2007; Segovia-Silvestre *et al.*, 2009).

A final theory suggests the local physical features of the bone surface following resorption may also play a role in directing subsequent bone formation. It is proposed that such a mechanism would explain why mechanical stimulation does not lead to new bone being formed at any type of surface, and it is cells interaction with the three-dimensional substratum at resorption sites that results in their stretching or deformation (Gray *et al.*, 1996). *In vitro*, pre-osteoblasts fill resorption pits until a flat plane is generated before bone formation begins. Additionally, once formation does commence, the rate of filling of pits is initially rapid, but slows as the pit filling nears and the level plane is restored (Jones *et al.*, 1994). However, as the pits had been created by osteoclasts, it is possible that this effect was due to other factors such as those derived from the resorbed tissue or the resorbing cell itself.

Subsequently, (Gray *et al.*, 1996) showed that when dentine slices with grooves of varying depth cut into them were seeded with rat calvarial osteoblasts and cultured together, in the first 18 days, 100% of new tissue was formed within the grooves. New tissue formation occurred earlier in deeper grooves compared to shallower ones, with bone formation tapering as a groove became shallower. The authors also observed that the depth of groove that appeared most advantageous for new bone formation was not dissimilar to the depth of a Howship's Lacunae (Gray *et al.*, 1996). The apparent importance of sufficient pit depth in attracting bone formation was confirmed by Gray in a subsequent study (Gray, 1998) suggesting that the tight regulation of osteoclast apoptosis might not only serve to limit the extent of bone resorption, but also to ensure that sufficient physical disruption (*i.e.* pit depth) of the bone surface occurs to differentiate it from the smooth surface around, ensuring bone formation occurs in the correct location.

2.1.1.5 Bone Formation

Bone formation is completed by osteoblasts that arise from the expansion of their precursors called osteoprogenitors that first differentiate into preosteoblasts and then on to mature bone-forming cells. This series of steps, from mesenchymal stem cells (MSC) to mature osteoblasts is controlled by the complex interplay between cytokines, hormones and their receptors. Key in the commitment of MSCs to the osteoblast lineage is runt-related transcription factor-2 (RUNX2) also known as cbfa1 (Otto *et al.*, 1997; Xiao *et al.*, 2004) while the transcription factor osterix is essential for the differentiation of preosteoblasts into mature osteoblasts (Nakashima *et al.*, 2002).

The source(s) of these precursor cells remains to be completely determined. With the identification of the sealed BRC, in order to explain the presence of osteoblasts inside it, Rasmussen and Bordier, (1974) and then Parfitt, (2001) suggested that the origin of preosteoblasts must be the cells of the BRC canopy itself, which are positive for a range of osteoblast markers (Hauge *et al.*, 2001; Andersen *et al.*, 2009) (Figure 2.1). The osteogenic potential of bone lining cells – from which these cells began – has been demonstrated previously, having been shown to revert back to osteoblasts when stimulated by intermittent PTH (Dobnig and Turner, 1997) or mechanical loading (Chow *et al.*, 1998).

However, the identification of the BRC as a vascular structure (Hauge *et al.*, 2001; Andersen *et al.*, 2009) provides a route by which pre-osteoclasts, like pre-osteoclasts, could migrate directly the BRC from external sources via the circulation (Figure 2.2).

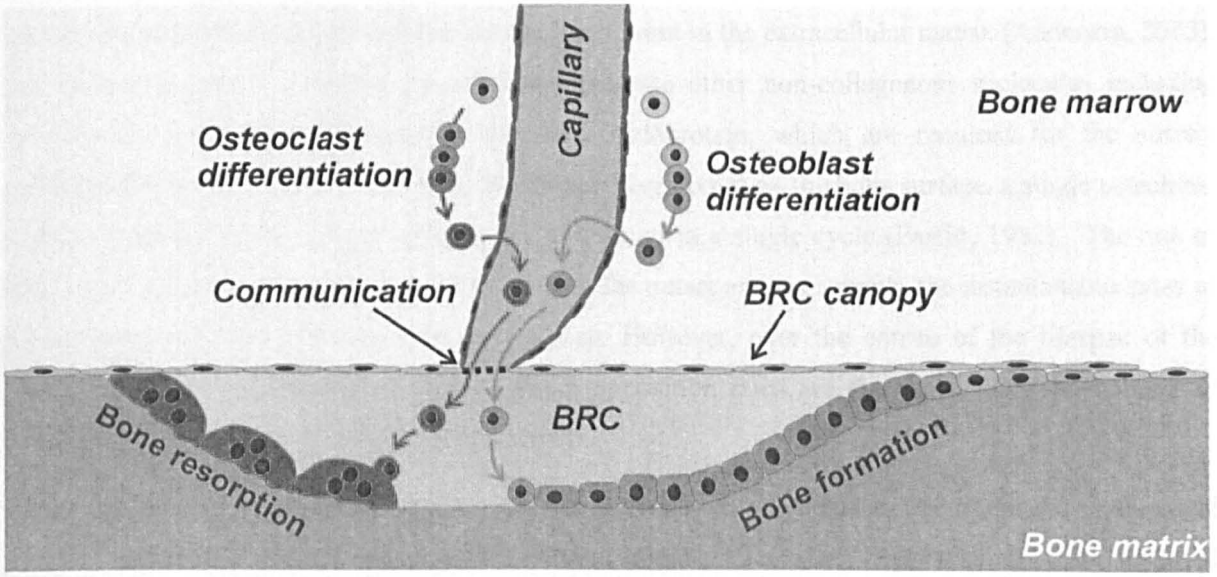

Figure 2.2. The vascular structure of a bone remodelling compartment (BRC) with a canopy of ostoblast lineage cells connected to capillaries separating the compartment from the bone marrow. The capillaries provide a mechanism by which both pre-osteoclasts and osteoblast progenitors can migrate into the BRC from external sources, although it remains to be elucidated where osteoblast progenitors are positioned and how the capillaries communicate with the BRC. (From Andersen *et al.*, 2009).

Importantly, Andersen *et al.* (2009) also showed that disruption of this canopy in multiple myeloma was associated with deficient bone formation. These findings strengthen the possibility that the circulation might be a source of pre-osteoclasts (Zvaifler *et al.*, 2000; Kuznetsov *et al.*, 2001; Eghbali-Fatourechi, 2005). A further possible source of pre-osteoblasts is adherent bone marrow stromal cells entering the BRC through diapedesis, although this would not account for osteoblastic cells in bone multicellular units in cortical bone.

Alternatively, bone marrow nonadherent osteoblastic cells could enter through the marrow microcirculation and then the peripheral circulation. Additionally, the localisation of BRCs with the vasculature indicates the possibility of resident cells in these vessels, such as pericytes, entering the BRCs and differentiating, as endothelial cells and osteoblasts may have a common origin (Doherty *et al.*, 1998; Minasi *et al.*, 2002). The latter two sources would account for the presence of osteoblasts in cortical bone BMUs.

Mature, bone-forming osteoblasts exist in heterogeneous clusters with bone lining cells. They express high levels of alkaline phosphatase (ALP) activity and firstly secrete type 1 collagen onto the bone surface which forms the organic, unmineralised matrix known as osteoid. Following the synthesis and secretion of the matrix, osteoblasts then trigger the mineralization of the matrix by releasing small membrane-bound vesicles, known as matrix vesicles that are responsible for establishing suitable conditions for mineral deposition. These conditions are achieved through release of appropriate concentration of calcium and PO_4 ions and the enzymatic degradation of inhibitors of mineralization such as pyrophosphate and proteoglycans that are present in the extracellular matrix (Anderson, 2003). In addition to type 1 collagen, osteoblasts synthesise other non-collagenous molecules including osteocalcin, osteopontin, osteonectin and bone sialoprotein, which are required for the correct mineralisation of the matrix. It is likely that at any given point on the bone surface, a single osteoblast is responsible for all the martix formation and does so in a single cycle (Parfitt, 1982). The rate of both matrix and mineral apposition are greatest at the outset and, as a result, the instantaneous rates of the two processes are systematically out of step. However, over the course of the lifespan of the osteoid seam, in the absence of disease, mean apposition rates are the same, with the volume of mineralised bone matching that of the new matrix.

The progressive decrease in apposition rates corresponds with osteoblasts undergoing morphological changes, becoming flatter and broader, which might reflect the beginning of their terminal differentiation into bone lining cells and the return of the bone surface to the quiescent state. In addition to differentiating into bone lining cells (~5%), like osteoclasts, osteoblasts also undergo apoptosis (~70%). Importantly, however, ~25% of osteoblasts are entombed in the newly formed bone as osteocytes. In mature bone, osteocytes are the most abundant cells. Due to their abundance, their distribution throughout the bone volume and their connections with bone lining cells, they are thought to act as a mechanosensor and transducer in bone and be involved in the detection and repair

of microcracks. In this model, the loss of viable osteocytes disturbs the osteocyte-canaliculi network resulting in a failure to detect microcracks and induce their repair (Noble, 2005).

2.2 Bone turnover markers

2.2.1 Bone resorption markers

Bone resorption can be assessed by several biochemical markers including products of type 1 collagen catabolism: hydroxyproline (Hyp), hydroxylysine (Hyl) glycoside, pyridinoline (PYD), deoxypyridinoline (DPD), the C-terminal cross-linking telopeptides of type 1 collagen generated by matrix metalloproteinases (CTX-MMP, 1CTP), the N-terminal and C-terminal cross-linking telopeptides of type I collagen (NTX and CTX), and helicoidal peptide 620-633 (HELP); non-collagenous proteins: bone sialoprotein (BSP) and osteocalcin (OC) fragments and; osteoclast enzymes: the isoform 5b of tartrate-resistant acid phosphatase (TRACP) and its isoform 5b (TRACP5b), and the cathepsins K and L (Table 2.1).

Both urinary DPD and serum 1CTP correlate with histomorphometric estimates of bone resorption (Eriksen *et al.*, 1993; Roux *et al.*, 1995), while plasma β-CTX correlates with estimates of bone formation during anabolic treatment (Recker *et al.*, 2009). Histomorphometric analysis in these studies was performed in biopsies taken from the iliac crest and so describes mainly trabecular bone remodelling in a small part of the skeleton. In contrast, calcium kinetic studies provide estimates of whole body resorption and formation rates representing the sum of cortical, endosteal and trabecular turnover. Estimates of bone resorption from radiotracer kinetic studies correlate with baseline levels of DPD and PYD but not Hyp (Eastell *et al.*, 1997) while the association between 1CTP and calcium kinetics is strong in myxedema, thyrotoxicosis and primary hyperparathyroidism (HPT) and weak in osteoporosis, but not present in healthy controls (Charles *et al.*, 1994). During antiresorptive treatment, the change in plasma CTX, but not DPD or PYD correlates with calcium kinetics (Denk *et al.*, 2007). Thus, these studies indicate that bone resorption markers are a reflection of whole body bone resorption rates.

2.2.1.1 Products of type 1 collagen catabolism

2.2.1.1.1 Hydroxyproline

Hydroxyproline is an amino acid that is present in all collagen types and tissues and constitutes 12-14% of the total amino acid content of mature collagen. Ninety percent of the Hyp liberated during the degradation of bone collagen is reabsorbed, further metabolized or reutilized for collagen synthesis (Lowry *et al.*, 1985) leaving the remaining 10% to be excreted in the urine. As well as collagen degradation, significant amounts of urinary Hyp are derived from the degradation of newly synthesised collagen (Smith, 1980). Hydroxyproline is also found in other tissues such as the skin and

is liberated from the metabolism of elastin and C1q (Prockop *et al.*, 1979). For these reasons, and its presence in foods containing gelatine, is has largely replaced by more specific bone resorption markers.

2.2.1.1.2 Hydroxylysine-glycosides

The hydroxylysine-glycosides occur in two forms, glycosyl-galactosyl-hydroxylysine (GGHL) and galactosyl-hydroxylysine (GHL) (Cunningham *et al.*, 1967). Hydroxylysine is preferable to Hyp as, in its glycosylated forms, it is not metabolised or influenced by dietary intake (Cunningham *et al.*, 1967; Moro *et al.*, 1984). Additionally, as GGHL is also present in skin and C1q, and GHL is more specific for bone, the ratio of GGHL/GHL may allow for the recognition of existent tissue specificity. Whilst potentially a useful marker of bone resorption, the routine measurement of hydroxylysine-glycosides is limited by the lack of a convenient immunoassay format.

2.2.1.1.3 3-Hydroxypyridinium crosslinks of collagen pyridinoline and deoxypyridinoline

PYD and DPD are formed during the extracellular maturation of fibrillar collagens, mechanically stabilising the molecule by cross-linking the telopeptide domain of a collagen fibril to the helical region of an adjacent collagen fibril (Fujimoto *et al.*, 1978). During bone resorption, PYD and DPD are released into the circulation in approximately a 3/4:1 ratio as free molecules or attached to collagen fragments, or peptide bound. Unlike Hyp, there is no evidence of their metabolism, they are unaffected by dietary collagen intake (Colwell *et al.*, 1993) and neither derivative is present in other sources such as skin (Boucek *et al.*, 1981). Additionally, again unlike Hyp, as cross links are only formed in the final stages of fibrillogenesis, the measurement of PYD and DPD is not influenced by the degradation of newly synthesised collagens. DPD is found almost exclusively in bone and dentin and, although PYD is found in cartilage, bone, ligaments and vessels, they both show a high specificity for skeletal tissues. This specificity, combined with a much higher turnover rate in bone compared with cartilage, ligaments, vessels or tendons, means that the amounts of PYD and DPD in blood or urine are mainly derived from the skeletal metabolism.

Analysis of urine shows that 40-50% of pyridinolines exist in the free form with the remaining 50-60% in the peptide-bound form (Seibel *et al.*, 1994). The acid hydrolysis of samples allows the measurement of both forms (total pyridinolines) by HPLC. Levels of free and total pyridinolines are reported to correlate well (Robins *et al.*, 1994; James *et al.*, 1996) but some different responses have also been observed. In both healthy women and those with metabolic bone disease, the fraction of free pyridinolines is negatively correlated with total pyridinoline excretion (Garnero *et al.*, 1995) a finding subsequently confirmed in children (Colwell and Eastell, 1996), suggesting a greater increase in the peptide-bound fraction with increased bone resorption.

Table 2.1. Biochemical markers of bone resorption. *Adapted from Seibel, (2005) and Seibel, (2006).*

Marker (abbreviation)	Tissue of origin	Analytical specimen	Analytical method	Remarks/specificity
Collagen-related markers				
Hydroxyproline, total and dialyzable (Hyp)	Bone, cartilage, soft tissue, skin	Urine	CM, HPLC	Present in all fibrillar collagens and partly collagenous proteins, including C1q and elastin. Present in newly synthesized and mature collagen (*i.e.* both collagen synthesis and tissue breakdown contribute to urinary hydroxyproline)
Hydroxylysine glycosides (Hyl)	Bone, soft tissue, skin, serum complement	Urine	HPLC	Hydroxylysine in collagen is glycosylated to varying degrees, depending on tissue type. Glycosylgalactosyl-OHLys in high proportion in collagens of soft tissues, and C1q; Galyctosyl-OHLys in high proportion in skeletal collagens
Pyridinoline (PYD)	Bone, cartilage, tendon, blood vessels	Urine, serum	HPLC, Tandem MS, ELISA	Collagens, with highest concentrations in cartilage and bone; absent from skin; present in mature collagen only
Deoxypyridinoline (DPD)	Bone, dentin	Urine, serum	HPLC, Tandem MS, ELISA	Collagens, with highest concentration in bone; absent from cartilage or skin; present in mature collagen only
Carboxyl-terminal cross-linked telopeptide of type 1 collagen (CTX-MMP, 1CTP)	Bone, skin	Plasma, serum	RIA	Collagen type 1, with highest contribution from bone; may be derived from newly synthesized collagen
Amino-terminal cross-linked telopeptide of type 1 collagen (NTX-1)	All tissues containing type 1 collagen	Urine, plasma, serum	ELISA, CLIA, RIA	Collagen type 1, with highest contribution from bone
Carboxyl-terminal cross-linked telopeptide of type 1 collagen (CTX-1)	All tissues containing type 1 collagen	Urine, plasma, serum	ELISA, RIA ECLIA	Collagen type 1, with highest contribution from bone. Isomerization of aspartyl to <beta>-aspartyl occurs with ageing of collagen molecule

Marker (abbreviation)	Tissue of origin	Analytical specimen	Analytical method	Remarks/specificity
Collagen 1 α-1 helicoidal peptide (HELP)	All tissues containing type 1 collagen	Urine	ELISA	Degradation fragment derived from the helical part of type 1 collagen (α-1 chain, AA 620-633). Correlates highly with other markers of collagen degradation, no specific advantage or difference in regards to clinical outcomes
Non-Collagenous Proteins				
Bone sialoprotein (BSP)	Bone, dentin, hypertrophic cartilage	Serum	RIA, ELISA	Acidic, phosphorylated glycoprotein, synthesized by osteoblasts and osteoclastic-like cells, laid down in bone extracellular matrix. Appears to be associated with osteoclast function
Osteocalcin (OC) fragments (ufOC, U-Mid-OC, U-LongOC)	Bone	Urine	ELISA, ECLIA, RIA	Certain age-modified OC fragments are released during osteoclastic bone resorption and may be considered an index of bone resorption
Osteoclast enzymes				
Tartrate-resistant acid phosphatase (TRACP)	Bone, blood	Plasma, serum	CM, RIA, ELISA	Six isoenzymes found in human tissues (osteoclasts, platelets, erythrocytes). Band 5b predominant in bone (osteoclasts). Enzyme identified in both the ruffled border of the osteoclast membrane and the secretions in the resorptive space
Cathepsins (*e.g.* K, L)	K – primarily osteoclasts; L – macrophages; osteoclasts	Plasma, serum	ELISA	Cathepsin K, a cysteine protease, plays an essential role in osteoclast-mediated bone matrix degradation by cleaving helical and telopeptide regions of collagen type 1. Cathepsin K and L cleave the loop domain of TRACP and activate the latent enzyme. Cathepsin L has a similar function in macrophages. Tests for measurement of cathepsins in blood are presently under evaluation.

CM, colorimetric; CLIA, chemoluminescence assay; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; MS, mass spectrometry; RIA, radioimmunoassay.

In patients receiving estrogen therapy, total, free and peptide-bound DPD decrease in concert (Garnero *et al.*, 1995) but with bisphosphonate therapy, free pyridinolines tend to be less responsive than total pyridinolines (Blumsohn *et al.*, 1995b; Garnero *et al.*, 1995; Tobias *et al.*, 1996; Naylor *et al.*, 2003a). Free pyridinolines are also less responsive to calcium supplementation (Rubinacci *et al.*, 1999). However, these different differences might be attributed to the effects of bisphosphonates on renal tubular PO_4 handling (Vasikaran *et al.*, 1994) and the different renal clearance of free and bound crosslinks (Colwell and Eastell, 1996; Naylor *et al.*, 2003a) respectively.

Regarding renal clearance, Colwell and Eastell, (1996) measured free pyridinolines in both serum and urine and observed that levels were 50% higher in urine, a finding subsequently confirmed by Naylor *et al.* (2003a). These findings suggest that a portion of the free pyridinolines in urine is produced in the kidney, an idea supported by animal studies that show the denaturing of type 1 collagen peptides reabsorbed at the proximal tubule brush border by lysosomal enzymes (Rucklidge *et al.*, 1988). As the percentage of free pyridinolines in urine appears to decrease with increasing bone turnover, Randall *et al.* (1996) proposed a model in which pyridinoline containing collagen fragments are sequentially degraded to free pyridinolines with a rate-limiting step in the sequence between peptide forms.

Of PYD and DPD, DPD is considered more specific to bone which is supported by data showing a greater reduction in DPD than PYD with bisphosphonate therapy in osteoporotic (Tobias *et al.*, 1996; Srivastava *et al.*, 2003) and cancer patients (Vinholes *et al.*, 1996).

2.2.1.1.4 The cross-linked telopeptide of type 1 collagen

The cross-linked telopeptides of type 1 collagen are derived from the amino terminal and the carboxyterminal telopeptide of the collagen type 1 molecule. On the N-telopeptide end, one fragment has been classified known as the cross-linked N-terminal telopeptide of type 1 collagen (NTX). On the C-telopeptide end, two fragments have been characterized, the first of which is cross-linked carboxyterminal telopeptide of type 1 collagen (1CTP) derived from the actions of matrix metalloproteinases and hence also known as CTX-MMP. The second fragment is the cross-linked carboxyterminal telopeptide of type 1 collagen (CTX) which, in turn, can be further classified according to structure of the collagen molecule from which it is generated (described below).

2.2.1.1.5 Cross-linked amino-terminal telopeptide of type I collagen

The assays that measure NTX uses a monoclonal antibody raised against an epitope on the α-2 chain of type 1 collagen and are able to measure NTX in urine (Hanson *et al.*, 1992) and serum (Clemens *et al.*, 1997). The antibody, however, reacts with several cross-linking components, and the presence of a pyridinium cross-link is not essential for reactivity. Extracts of skin collagen also exhibited similar reactivity with the NTX assay as skeletal extracts (Robins, 1995). The monoclonal antibody and the

assay format are the same for the urine and the serum assays and show good correlation with each other in cross-sectional studies of pre- and postmenopausal women and patients with Paget's disease, and in the response to antiresorptive therapy in Paget's patients (Clemens *et al.*, 1997). Serum NTX is better than urinary NTX at differentiating between healthy controls and patients with primary vertebral osteoporosis and primary HPT (Woigte *et al.*, 1999). The serum assay also has lower short- and long-term intra-subject variability, although the two assays have similar signal-to-noise ratios with hormone replacement therapy (Eastell *et al.*, 2000). However, there is recent evidence that urinary NTX displays a more rapid (4 weeks *vs* 16 weeks for serum NTX) response to bisphosphonate therapy and has a higher signal to-noise-ratio (Abe *et al.*, 2008). The more rapid reduction in urinary NTX with antiresorptive therapy may improve patient adherence to treatment (Clowes *et al.*, 2004).

2.2.1.1.6 Cross-linked carboxyterminal telopeptide of type 1 collagen.

Immunoassays of serum cross-linked carboxyterminal telopeptide of type 1 collagen generated by matrix metalloproteinases (MMP), referred to as 1CTP, detect the telopeptide portion of the collagen fragment that resides between the 2 alpha-1 chains (Sassi *et al.*, 2000) with the antigenic determinant requiring the presence of a trivalent cross-link, including two phenylalanine-rich domains. These assays benefited from an antibody that shows no reaction with divalently and non-cross-linked peptides or with peptides isolated from skin. Although initially 1CTP was thought to hold promise as a specific marker of bone resorption, Sassi *et al.* (2000) showed it to be generated by the actions of matrix metalloproteinases (MMP) with MMP-mediated osteoclastic bone resorption leaving the antigenic site intact. In contrast, cathepsin K destroyed 1CTP antigenicity by cleaving the collagen at the antigenic site (between F [phenylalanine] and L [leucine] as well as between Q [glutamine] and E [glutamic acid]) (Sassi *et al.*, 2000). Although 1CTP is destroyed by cathepsin K (Sassi *et al.*, 2000), this enzyme is efficient at generating CTX, the other cross-linked carboxyterminal telopeptide of the type 1 collagen fragment (Figure 2.3).

The CTX fragment can be further classified according to structure of the collagen molecule from which it is generated. As type 1 collagen ages in the extracellular matrix, two spontaneous transformations occur, so-called racemization and isomerisation (Fledelius *et al.*, 1997; Cloos and Fledelius, 2000) and hydrolysis of the type 1 collagen molecule generates four different isoforms: the native peptide isoform, (α-L C-terminal cross-linking telopeptide of type 1 collagen - CTX) and three age-related isoforms, the isomerized α β-L-CTX (β-CTX), the racemised (α-D-CTX), and the isomerised *and* racemised form (β-D-CTX). These age-related spontaneous transformations can be detected *in vivo* by measuring the urinary excretion of the corresponding CTX using specific antibodies (Cloos and Fledelius, 2000).

These fragments have been used to study the association between modifications of type 1 collagen structure and reduced bone strength first indicated from the study of osteogenesis imperfecta and other clinical syndromes attributable to mutations of type 1 collagen genes (Boskey *et al.*, 1999). A retrospective, cross-sectional study of patients with fractures reported a discordance between changes in plasma α-L- and β-L-CTX and the resulting increase in the α-L/β-L-CTX ratio (Hoshino *et al.*, 1999), suggesting that the degree of type 1 collagen isomerisation also could be altered in patients with osteoporosis.

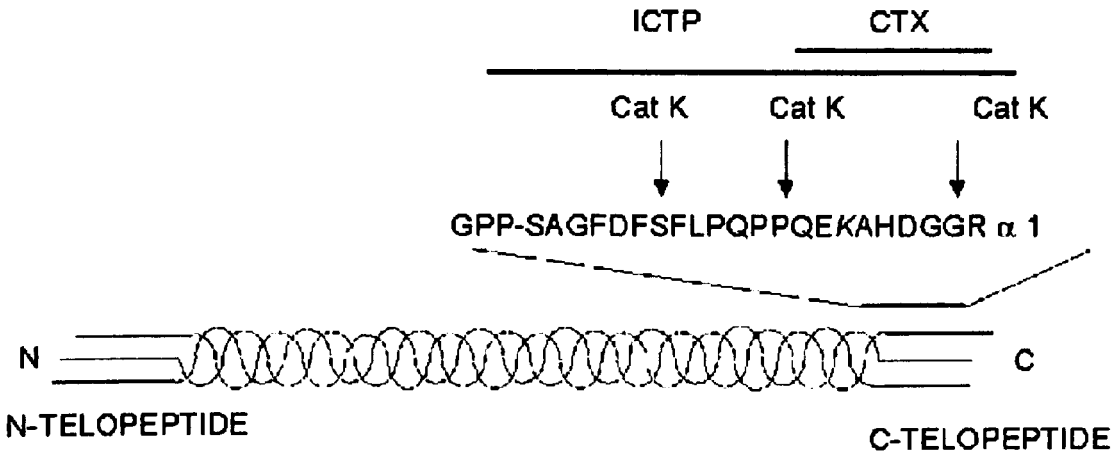

Figure 2.3. Schematic representation of the CTX and 1CTP epitopes used as markers of bone resorption on type 1 collagen and cathepsin K (Cat K) cleavage sites. CTX epitope is constituted by an eight amino acid sequence on the C-telopeptide of α1. The 1CTP epitope is a larger conformational epitope including at least two telopeptides and the first phenylalanine of the phenylalanine rich region. In generating CTX, cleavage by cathepsin K degrades the 1CTP epitope. *From Garnero et al. (2003b).*

In a large prospective study (Garnero *et al.*, 2002), the decreased degree of type 1 collagen racemisation/isomerisation, as measured by urinary (u)CTX ratios, in untreated postmenopausal women is associated with increased fracture risk independent of BMD and partly of bone turnover rate. This suggests that alterations of type 1 collagen modification may be associated with increased skeletal fragility, which is supported by more recent studies that show the extent of post-translational modifications contributes to the bending and biomechanical properties of fetal bovine cortical bone (Garnero *et al.*, 2006; Viguet-Carrin *et al.*, 2006). The α-CTX/β-CTX ratio in dog vertebra is decreasaed following bisphosphonate therapy possibly explaining some of the anti-fracture efficacy of this type of therapy (Allen and Burr, 2008). Interestingly, in osteoporotic women, this ratio is increased following 12 months of PTH 1-84 therapy followed by either 12 months of bisphosphonates or placebo suggesting PTH therapy might result in decreased isomerisation, possibly resulting from a decrease in mean tissue age as a result of remodelling (Garnero *et al.*, 2008a).

Paget's patients, who have high levels of localised bone resorption and formation and abnormal woven bone characterised by reduced strength, were shown to have a greater increase in plasma α-CTX than β-CTX resulting in a 3-fold increase in the α-CTX/β-CTX ratio (Garnero *et al.*, 1997). This ratio returns to within the normal range with bisphosphonate therapy (Garnero *et al.*, 1998; Peris *et al.*, 2002) while plasma α-CTX shows a more marked reduction compared with other bone turnover markers (Alexandersen *et al.*, 2005). Plasma α-CTX may also be a useful marker of both disease activity in these patients (Alexandersen *et al.*, 2005). A comparison of Paget's patience with those with primary HPT, who also have increased bone turnover (although not to the same degree as in Paget's) but show no evidence of alterations of bone matrix structure and have normal lamellar bone, provides an insight into the importance of post-translational changes in bone structure. In contrast to Paget's patients, Garnero *et al.* (1997) report a marked increase in both plasma α-CTX and β-CTX resulting in an unaltered α-CTX/β-CTX ratio in patients with primary HPT. These findings suggest that the defect in isomerisation may be observed only with very high bone turnover that results in the formation of woven bone, and the increased α-CTX/β-CTX ratio in these patients reflects the degradation of woven bone with a low degree of β-isomerisation (Garnero *et al.*, 1997). The decrease in this ratio following treatment (Garnero *et al.*, 1998; Peris *et al.*, 2002) may well reflect the return to the formation of lamellar bone (Meunier *et al.*, 1980).

uβ-CTX measurements have poor precision at concentrations lower than 200 $\mu g \cdot L^{-1}$ and, as plasma β-CTX performs as well as uβ-CTX in reflecting the increase of bone resorption in patients with vertebral fractures and hip fractures (Kawana *et al.*, 2002), the response to antiresorptive treatment (Fall *et al.*, 2000) and in predicting hip fracture, the measure of plasma β-CTX may be preferable. A plasma sample also eliminates the imprecision of a uβ-CTX sample that exists due to variable urine dilution and by removing the necessity for a correction factor (*e.g.* creatinine).

2.2.1.1.7 1CTP vs CTX as a marker of bone resorption

The destruction of 1CTP by cathepsin K (Sassi *et al.*, 2000) but its efficient generation of CTX suggests that these fragments are released through distinct collagenolytic pathways (Garnero *et al.*, 1998) (Figure 2.2.1). This was confirmed by Garnero *et al.* (2003b) who showed the release of CTX but not 1CTP by cathepsin K, and 1CTP but not CTX with MMP-2, MMP-9, MMP-13, or MMP-14. Importantly cathepsin K inhibitors inhibit the release of CTX, but not 1CTP, while inhibitors of MMPs inhibited the release of 1CTP and CTX (Garnero *et al.*, 1998).

Different collagenolytic pathways provides an explanation as to why levels of 1CTP and CTX vary with different pathologies and in response to treatment. For example, plasma β-CTX is increased in postmenopausal, osteoporotic women (Garnero *et al.*, 1994a; Garnero *et al.*, 1994b; Guerrero *et al.*, 1996; Rosenquist *et al.*, 1998), and β-CTX levels are reported to be efficient markers of bone resorption in metabolic bone diseases, characterized by increased bone resorption, including

osteoporosis, Paget's disease, primary HPT and hyperthyroidism (Garnero *et al.*, 1994a). In contrast, 1CTP remains unchanged in postmenopausal women (Garnero *et al.*, 1994b; Guerrero *et al.*, 1996). In Cushing's syndrome, compared to healthy controls, uβ-CTX is decreased 32% whereas there is no difference in 1CTP (Cortet *et al.*, 2001).

β-CTX and 1CTP also respond differently according to the clinical situations and treatments. For example, in post-menopausal women undergoing antiresorptive therapy, plasma β-CTX concentrations are markedly decreased (Garnero *et al.*, 1994b; Delmas *et al.*, 1997; Christgau *et al.*, 1998; Ravn *et al.*, 1999; Christgau *et al.*, 2000) whereas 1CTP remains unchanged (Garnero *et al.*, 1994b). Plasma β-CTX is also a good predictor of gain of spinal BMD in patients treated with bisphosphonates (Christgau *et al.*, 1998; Christgau *et al.*, 2000; Fink *et al.*, 2000) and HRT (Christgau *et al.*, 1998; Christgau *et al.*, 2000). In prostate cancer patients treated with zoledronate, plasma β-CTX shows a marked decrease whereas 1CTP is unchanged (Lein *et al.*, 2007).

In RA patients treated with HRT, plasma β-CTX declines significantly (Forsblad d'Elia *et al.*, 2004) whereas 1CTP shows only a small (5%) decrease (Forsblad d'Elia *et al.*, 2004) or is unchanged (Lems *et al.*, 1993). Additionally, although the decrease in both plasma β-CTX and 1CTP were associated with improved bone mass after 2 years of HRT, CTX provided the most sensitive prognostic value (Forsblad d'Elia *et al.*, 2004). Similarly, postmenopausal women undergoing HRT show a marked decrease in plasma β-CTX but no change in 1CTP (Hassager *et al.*, 1994; Rosenquist *et al.*, 1998). This is in line with previous reports in which 1CTP has shown only a very limited response to bisphosphonate in osteoporotic women (Pedrazzoni *et al.*, 1995) and Paget's patients (Blumsohn *et al.*, 1995b; Pedrazzoni *et al.*, 1995). Plasma β-CTX is also better than 1CTP in predicting changes in BMD following treatment with HRT (Okabe *et al.*, 2004).

Surprisingly, in a study of elderly community-dwelling men, 1CTP levels but not plasma β-CTX levels were significantly elevated in men with subsequent fractures and only 1CTP was predictive for incident fractures (Meier *et al.*, 2005). This finding, however, might have been influenced the lack of an enforced fast prior to blood sampling that would have affected plasma β-CTX concentrations (See *Pre-analytical variability in urinary bone turnover markers*). Finally, plasma β-CTX and 1CTP also respond differentially to non-pharmacological interventions, with β-CTX but not 1CTP suppressed for several hours following the acute ingestion of calcium (Guillemant *et al.*, 2003) and macronutrients (Hassager *et al.*, 1992).

In contrast, in multiple myeloma, that is associated with osteolytic bone lesions, 1CTP concentrations are increased (Elomaa *et al.*, 1992; Abildgaard *et al.*, 2000) and correlate with histomorphometric indices of bone resorption (Abildgaard *et al.*, 2000). Furthermore, the changes of 1CTP levels correlate with the clinical response in these patients during systemic therapy (Blomqvist *et al.*, 1996; Koga *et al.*, 1999). 1CTP is also a sensitive marker to detect osteolysis related to bone metastasis

from breast (Blomqvist *et al.*, 1996; Berruti *et al.*, 1999), prostate (Kylmälä *et al.*, 1995; Maeda *et al.*, 1997) and lung (Kong *et al.*, 2007) cancers. This increase may well reflect the increased activity of MMPs, which originate from malignant and non-malignant cell populations that increase MMP-mediated bone resorption and result in dysregulation of bone turnover at the site of tumour invasion. This resorption may in turn release factors which further stimulate tumour cell growth (Woodward *et al.*, 2007). Importantly, 1CTP is also elevated following bone fracture suggesting the differential diagnosis of bone fragility fracture and bone metastasis using 1CTP at the same time may be difficult, especially from a single sample (Takahara *et al.*, 2007).

There is increasing evidence that acidification-independent MMP activity, and thus 1CTP levels are increased in situations where acidification-dependent bone resorption is deficient (Henriksen *et al.*, 2006) suggesting that in some way MMPs compensate for the absence of cathepsin K. Thus, in patients with pycnodysostosis, β-CTX is decreased but 1CTP is increased (Nishi *et al.*, 1999) while serum 1CTP is increased in cathepsin K knock-out mice (Kiviranta *et al.*, 2005). In summary, plasma β-CTX appears to be superior to 1CTP in reflecting changes in cathespin K-medicated bone resorption, such as in conditions like osteoporosis, whilst in metastatic disease, or where cathespin K activity is compromised, collagen is mainly degraded by MMPs and this is reflected in the 1CTP concentration.

2.2.1.1.8 Helicoidal peptide 620-633

During bone resorption, type 1 collagen is cleaved in both the helicoidal and telopeptide regions. Helicoidal peptide 620-633 is a type 1 collagen-specific peptide corresponding to residues 620 to 633 of the helicoidal region of the α1 chain, for which ELISA assays have been developed (Ju *et al.*, 1997; Garnero and Delmas, 2003a). Helicoidal peptide is higher in postmenopausal women compared with premenopausal women, correlates with uβ-CTX, and is as sensitive as plasma β-CTX to assess the effects of antiresorptive therapy on BMD in postmenopausal women (Garnero and Delmas, 2003a). As changes in helicoidal peptide mirror those of plasma β-CTX, a marker considered to reflect cathepsin K-mediated bone resorption, it is possible that helicoidal peptide reflects the same mechanism. This appears to be supported by experiments showing that cathepsin K cleaves type 1 collagen molecules in both the telopeptides and within the triple helix (Garnero *et al.*, 1998). However, there remains very little additional published literature on the helicoidal peptide as a marker of bone resorption to verify this finding.

2.2.1.2 Non-collagenous proteins

2.2.1.2.1 Bone sialoprotein

Bone sialoprotein is a non-collagenous bone matrix protein and accounts for 5 to 10% of the non-collagenous matrix of bone. It is synthesised by active osteoblasts and odontoblasts, but is also found in osteoclast-like and malignant cell lines and is present in all mineralized tissues. Bone sialoprotein promotes the adhesion of osteoclasts to the bone surface (Ross *et al.*, 1993) and may play a role in the nucleation of hydroxyapatite in the bone matrix (Nagata *et al.*, 1991). *In vitro*, BSP inhibits the differentiation of new osteoclasts (Raynal *et al.*, 1996) but in the presence of RANKL, might synergistically induce osteoclastogenesis (Valverde *et al.*, 2005).

Serum BSP levels are measured by immunoassays and antibodies do not cross-react with non-collagenous proteins such as osteonectin, fibronectin or osteocalcin (Karmatschek *et al.*, 1997). However, BSP makes a complex with factor H in serum that must be disrupted to accurately measure total BSP levels (Fedarko *et al.*, 2001). In postmenopausal women, BSP is higher compared with premenopausal women (Seibel *et al.*, 1996) and correlates with other markers of bone turnover (Störk *et al.*, 2000). Bone sialoprotein is also higher in primary HPT, Paget's disease, untreated multiple myeloma and breast cancer with bone metastases compared to healthy controls and correlates with urinary PYD and DPD in primary HPT (Seibel *et al.*, 1996). In patients with metastatic bone cancer, intravenous (iv) bisphosphonate treatment reduces BSP levels by 60% within four days of treatment (Seibel *et al.*, 1996) and is reduced approximately 50% after 1 year of HRT (Störk *et al.*, 2000). These data indicate that BSP appears to be a sensitive marker of bone turnover and that its serum levels predominantly reflect processes related to bone resorption.

2.2.1.3 Osteoclast enzymes

2.2.1.3.1 Tartrate-resistant acid phosphatase

Tartrate-resistant acid phosphatase (TRACP) belongs to a family of six isoenzymes (types 0–5) of acid phosphatases that are expressed by different tissues and cells including prostate, bone, spleen, platelets, erythrocytes, and macrophages. Of the six, only TRACP type 5 is not inhibited by L(+)- tartrate. Prior to specific immunoassays for total TRACP 5b, which measured both 5a and 5b, suggested that it was associated with bone turnover, but it performed poorly compared with several other known markers (Hannon *et al.*, 1998). The subform 5b (TRACP 5b) is characteristic of osteoclasts (Minkin, 1982) and is synthesized and secreted by osteoclasts during active bone resorption (Halleen *et al.*, 1996). Serum TRACP 5b is elevated in patients with bone diseases (Halleen *et al.*, 2001), correlates with other markers of bone turnover and BMD (Halleen *et al.*, 2002) and decreases after antiresorptive treatment, correlating with changes in other bone resorption markers (Nenonen *et al.*, 2005) and predicts future fractures (Gerdhem *et al.*, 2004).

Unlike markers such specific markers of osteoclast activity such as β-CTX, serum TRACP 5b appears to reflect osteoclast number. This is supported by several lines of evidence including the correlation of serum TRACP 5b with histomorphometrically determined osteoclast number from bone biopsies of patients with renal bone disease (Chu *et al.*, 2003), of TRACP 5b activity secreted into a culture medium with the number of non-resorbing multinucleated osteoclasts formed from bone marrow-derived osteoclast precursor cells (Alatalo *et al.*, 2000) and studies of osteopetrotic rat strains and human patients of type-2 autosomal dominant osteopetrosis (ADO2) that showed elevated TRACP 5b concomitantly with elevated numbers of functionally-inactive osteoclasts (Atalato *et al.*, 2003; Atalato *et al.*, 2004). More recently, in OVX animals, TRACP 5b has been shown to decrease and correlate with the number of osteoclasts/bone perimeter and number of osteoclasts/tissue (Rissanen *et al.*, 2008). In this study plasma β-CTX increased and the β-CTX to TRACP 5b ratio increased further still suggested that the ratio reflects the high resorbing activity of osteoclasts with estrogen withdrawal. This is consistent with the idea that TRACP 5b might be used in conjunction with β-CTX to obtain an index reflecting the mean activity of an individual osteoclast (Nenonen *et al.*, 2005).

The idea of TRACP 5b as a marker of osteoclast number, and also the potential benefit of using TRACP 5b and β-CTX in conjunction *in vitro*, has recently been supported by Rissanen *et al.* (2009). They showed a strong correlation between microscopically-counted osteoclasts and TRACP 5b concentrations in osteoclast differentiation cultures treated with differentiation inhibitors (OPG and alendronate). They also showed that by normalising β-CTX values for TRACP 5b values in an osteoclast activity culture treated with activity inhibitors (cysteine protease inhibitor E64 and cathespin K inhibitor ORG-29762), the significant and dose-dependent decrease in β-CTX was a result of a reduced number of osteoclasts rather than due to the concentrations of the inhibitors. Thus, this method prevents different numbers of osteoclasts in different wells confounding results from tests of antiresorptive compounds (Rissanen *et al.*, 2009).

Although serum TRACP 5b appears to reflect osteoclast number and thus be a useful marker of response to antiresorptive therapies that inhibit osteoclast differentiation and promote apoptosis, its value in Paget's disease is unclear. *In vitro*, pagetic osteoclasts form an increased number of osteoclasts which have more nuclei per cell and an increased resorption capacity compared to normal osteoclasts, while Paget's patients have localised regions of high bone turnover where new bone is architecturally disorganised, more vascular and less structurally sound. However, despite the increased resorption activity being reflected in increased β-CTX (3-fold) and especially α-CTX (16-fold) – with the later reflecting the resorption of immature collagen resulting from the increased rate of new bone formation – serum TRACP is only increased by 30% and, unlike, markers of osteoclast activity, TRACP shows no association with disease activity (Alexandersen *et al.*, 2005). Additionally, following bisphosphonate therapy, which both inhibits osteoclast differentiation and increases osteoclast apoptosis, both β-CTX and α-CTX are markedly decreased after 1 and 6 months

reflecting the osteoclast inhibition, whereas TRACP is actually significantly increased after 6 months. These findings might suggest that the association between serum TRACP and osteoclast number might be less strong where changes in bone metabolic activity are localised rather than systemic.

2.2.1.3.2 Cathepsin K

Unlike other members of the cysteine protease family, cathepsin K has the unique ability to cleave both helical and telopeptide regions of collagen 1 (Kafienah *et al.*, 1998; Li *et al.*, 2004). It is located throughout the cytoplasm of osteoclasts, appears at the ruffled border of actively resorbing osteoclasts and is secreted into bone resorption lacunae for extracellular collagen degradation (Goto *et al.*, 2003). Using a recently developed ELISA, it has been shown that cathepsin K levels are significantly higher in postmenopausal women with osteoporosis (Munoz-Torres *et al.*, 2009) and non-traumatic fractures (Holzer *et al.*, 2005) compared with healthy controls. Additionally, alendronate therapy resulted in a gradual decrease in cathepsin K levels over a 12 month period compared to a rapid (within 1 month) and sustained reduction in plasma β-CTX (Munoz-Torres *et al.*, 2009). As β-CTX is a measure of osteoclast activity (rather than number), this different pattern in response might indicate that cathepsin K reflects a different aspect of osteoclast biology although this remains to be clarified. As cathepsin K activates TRACP by excising the repressive loop subsequent to secretion of monomeric TRACP precursor into the acidic ruffled border area (Ljusberg *et al.*, 2005), it is possible that, like TRACP, cathepsin K levels may also reflect osteoclast number.

Although much less abundant in the active, resorbing osteoclast than cathepsin K (Drake *et al.*, 1996), there is also some evidence that cathepsin L might be a useful marker of bone resorption. Serum cathepsin levels are negatively associated with bone density and, like other markers of bone resorption, are markedly reduced with antiresorptive therapy (Lang *et al.*, 2004).

2.2.2 Bone formation markers

Bone formation can be assessed by biochemical markers including N-terminal and C-terminal propeptides of type 1 procollagen (P1NP, P1CP), total and bone-specific alkaline phosphatase (total ALP and bone ALP) and osteocalcin (OC) (Table 2.2). Plasma/serum levels of P1CP (Eriksen *et al.*, 1993) and OC (Delmas *et al.*, 1986) correlate positively with histomorphometric parameters of bone formation in a range of metabolic bone diseases, while serum bone ALP correlates with parameters of bone resorption and formation in dialysis patients (Ureña *et al.*, 1996). More recently, changes in P1NP and bone ALP have been shown to correlate with parameters of bone formation in response to treatment with anabolic drugs (Recker *et al.*, 2009). Like bone resorption markers, bone formation markers including total ALP (Parfitt *et al.*, 1987), OC (Charles *et al.*, 1985; Eastell *et al.*, 1988; Wand *et al.*, 1992) and P1CP (Parfitt *et al.*, 1987; Charles *et al.*, 1994) correlate with radiotracer kinetic estimates ($r = 0.49$ to 0.79), while changes in P1NP correlate ($r = 0.56$) with changes in

calcium kinetics during antiresorptive treatment (Denk *et al.*, 2007) indicating that bone formation markers accurately reflect whole body bone formation rates.

2.2.2.1 Propeptides of procollagen type 1

An important step in the formation of type 1 collagen is the enzymatic regulation by procollagen C-proteinase of the cleavage of P1CP and P1NP from procollagen to form insoluble collagen (Kessler *et al.*, 1996). Both N-terminal (P1NP) and C-terminal (P1CP) propeptides of procollagen type 1 are cleaved during the extracellular metabolism of procollagen and released into the blood, while the central portion of the molecule is incorporated into the bone matrix. Although neither P1NP nor P1CP are specific to bone, compared with other tissues from which they may originate, bone has a faster metabolism and the majority of serum P1NP and P1CP are released from bone. P1CP correlates weakly with histological bone formation in patients with vertebral osteoporosis (Parfitt *et al.*, 1987), although a good correlation is reported between the decrease in P1CP and the decrease in total ALP and bone ALP in osteoporotic patients treated with estrogens (Hasling *et al.*, 1991).

In contrast to P1CP, which is a single protein, P1NP circulates in several forms including the intact, authentic trimeric form, a monomer and several fragments (Brandt *et al.*, 1999). At physiological temperatures, there is a transition of the native P1NP to its monomeric form (Brandt *et al.*, 1999). Thus, assays for P1NP can be separated into those that measure both trimeric and the monomeric form (Total P1NP) and those that measure only the trimeric form (intact P1NP). The first isolation of the P1NP molecule from humans came with the identification of FA2 as a homomer of the α1 chain of the N-terminal propeptide of human procollagen type 1 [P1NP(ctl)] (Teisner *et al.*, 1992). Quantification of P1NP was performed by electroimmunoassay (Rasmussen *et al.*, 1992) and an inhibition enzyme-linked immunosorbent assay (ELISA) based on the inhibition of binding of biotin labelled rabbit anti-P1NP(al) to solid phase coupled P1NP(ct 1) in microtiter plates (Rasmussen *et al.*, 1992). The detection limit of the inhibition ELISA, however, was too high to study the parallelism between P1NP in normal human serum and the calibrator and to analyze the molecular distribution of P1NP in normal human serum (Rasmussen *et al.*, 1992). Subsequently, P1NP was also quantified by sandwich ELISA using purified α 1-chain specific rabbit antibodies (Ørum *et al.*, 1996). Parallelism was found between a calibrator, normal and patient serum, and purified P1NP (α 1), as well as the high and low molecular weight forms of P1NP (α 1) (Ørum *et al.*, 1996).

Table 2.2. Biochemical markers of bone formation. *Adapted from Siebel, (2007).*

Marker (abbreviation)	Tissue of origin	Analytical specimen	Analytical method	Remarks/specificity
Total alkaline phosphatase (total ALP)	Bone, liver, intestine, kidney, placenta	Plasma, serum	CM	Attached to the extracellular surface of cell membranes; may play a role in matrix mineralization; specific for bone formation only in the absence of liver or biliary disease
Bone-specific alkaline phosphatase (bone ALP)	Bone	Plasma, serum	CM, EP, precipitation, IRMA, EIA	Attached to the extracellular surface of cell membranes; may play a role in matrix mineralization; Specific product of osteoblasts; Carbohydrate side-chain differences confer bone specificity; Some assays show up to 20 % cross-reactivity with liver isoenzyme
Osteocalcin (OC)	Bone, platelets	Plasma, serum	RIA, ELISA, ECLIA	Contains calcium-binding amino acid and <gamma>- carboxyglutamic acid, which facilitates interaction with hydroxyapatite. Specific product of osteoblasts; many immunoreactive forms in blood; some may be derived from bone resorption
Carboxy-terminal propeptide of type 1 procollagen (P1CP)	Bone, soft tissue, skin	Plasma, serum	RIA, ELISA, HPLC	Specific product of proliferating osteoblasts and fibroblasts
Amino-terminal propeptide of type 1 procollagen (P1NP)	Bone, soft tissue, skin	Plasma, serum	RIA, ELISA, ECLIA, HPLC	Specific product of proliferating osteoblast and fibroblasts; partly incorporated into bone extracellular matrix

CM, colorimetric; EM, electrophoretic; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; IRMA, immunoradiometric assay; RIA, radioimmunoassay.

The ELISA assay used by Ørum *et al.* (1996) measured total P1NP (trimeric and monomeric forms as well as fragments) whereas a subsequent RIA assay (Melkko *et al.*, 1996) detected only the high molecular weight, trimeric form and as a result produced lower P1NP values than the ELISA in a patient with uremic hyperparathyroidism (Jensen *et al.*, 1998). The intact P1NP assay does not measure the small molecular weight degradation products that likely result from the degradation of so called pN-collagen (Jensen *et al.*, 1993a; Jensen *et al.*, 1993b). P1NP is found on the surface of newly-formed collagen fibres in soft tissues, but there is no type 1 pN-collagen in the mineralized bone matrix (Risteli and Risteli, 1995). As part of the P1NP formed in soft tissues might, during normal turnover, be degraded directly into fragments which are not recognized by the intact P1NP assay, the intact molecule may better reflect bone collagen synthesis. A further benefit of its specificity for the intact molecule only is that, due to its size, it is not subject to glomerular filtration removing any effect of alterations in kidney function on its concentrations. Recently a fully automated assay for intact P1NP in both its trimeric and monomeric forms had been described that produced higher P1NP values than the RIA, but exhibited high analytical reproducibility, was robust with respect to sample storage conditions (Garnero *et al.*, 2008b). It also documented a 73% increase in P1NP concentrations with menopause and changes in concentrations in excess of the least significant change (LSC) in 83% and 88% of patients after 3 months of PTH and alendronate therapy respectively (Garnero *et al.*, 2008b).

Several studies have compared P1NP and P1CP. Using an ELISA assay, Ørum *et al.* (1996) examined the sera of patients with hyperparathyroidism due to hypovitaminosis D and found significantly higher concentrations of P1NP compared with healthy controls, something not previously achieved with P1CP in these patients (Eriksen *et al.*, 1993). In healthy subjects, higher concentrations of P1NP compared with P1CP have been reported in children (Tähtelä *et al.*, 1997) and both healthy adult females and those with untreated postmenopausal osteoporotic women (Dominguez Cabrera *et al.*, 1998). P1NP outperformed P1CP in discriminating between osteoporotic and control women sensitivity and, regarding sensitivity and specificity, along with bone ALP, P1NP produced the best results among all bone formation markers studied (Dominguez Cabrera *et al.*, 1998). Menopause results in a greater increase in P1NP compared with P1CP (47.1 % *vs* 21.6 %) (Suvanto-Luukkonen *et al.*, 1997). One year treatment with percutaneously administered estradiol decreased P1NP by 42% compared to only 20% for P1CP (Suvanto-Luukkonen *et al.*, 1997). In this study, compared to P1CP, the change in P1NP with treatment allowed twice the number of patients to be correctly classified with regard to their change in spinal BMD, suggesting that the effect of estrogen on bone formation is better observed with P1NP than with P1CP. In osteoporotic women receiving four months of antiresorptive therapy (alendronate), a reduction in P1NP concentration in excess of the least significant change was observed in 75% of patients compared with only 10% of patients for P1CP (Fink *et al.*, 2000).

As, theoretically, P1NP and P1CP are formed in equimolar concentrations, differences between the behaviour of P1CP and P1NP could be related to differences in their release from procollagen in the tissues, the assay used, or in the process by which they are cleared from the circulation. There is a difference in the cleavage times of the two propeptides from the type 1 procollagen molecule. P1CP is liberated shortly after procollagen synthesis, and no functional collagen fibres can be formed if the P1CP remains attached to the collagen molecule (Miyahara *et al.*, 1982). In contrast, P1NP can be found on the surface of thin collagen fibres, where its presence prevents – and its rate of removal might regulate – the further growth of the fibre (Fleischmajer *et al.*, 1990).

It is known that the receptors involved in the clearance of these propeptides are different, with P1NP being cleared via scavenger liver receptors (Melkko *et al.*, 1994) and P1CP through mannose receptors of the cells (Smedsrod *et al.*, 1990). Whereas the expression of the scavenger receptor is apparently independent of hormones, the expression of the mannose receptor can be regulated by several factors including calcitriol, dexamethasone, and prostaglandin E (Shepherd *et al.*, 1985; Clohisy *et al.*, 1987; Schreiber *et al.*, 1990). Thus the elimination rate of P1NP from the circulation is less prone to variation than that of P1CP. Finally, Sorva *et al.* (1994) report a family with exceptionally high circulating P1CP concentrations but with normal P1NP levels.

As mentioned previously, compared with other tissues from which type 1 collagen may originate, bone has a faster metabolism, resulting in the majority of P1NP and P1CP in the circulation of resting, healthy humans representing that released from bone. When considering the effects of acute exercise, however, it is important to consider the acceleration in collagen metabolism in tissues other than bone and how that might contribute to P1NP levels.

Using microdialysis, Langberg *et al.* (1999) showed that the level of P1CP in the peritendinous tissue surrounding the Achilles tendon is decreased in the first 2 h after a 36 km run, but subsequently was increased 192% by 72 h post-exercise. This pattern of change was similar to that in plasma although the magnitude of the increase was comparatively modest (~25%). The dissociation between changes in P1CP in tissue and plasma was shown again by Olesen *et al.* (2007) who, following an identical exercise protocol, report increased tissue P1CP at 72 h and 96 h post-exercise while plasma levels remained unchanged. This dissociation is further supported by Heinemeier *et al.* (2003) who were unable to detect P1CP in the peritendinous tissue of the Achilles tendon until 68 h after 60 min of 3% uphill treadmill running whereas plasma concentrations were decreased 6 h after exercise but not different from pre-exercise levels thereafter. Importantly, in the study of Olesen *et al.* (2007), P1CP was also increased in a non-exercising control group at 72 h but not at 96 h indicating that the changes in P1CP at 96 h in the exercise group were likely due to exercise rather than to the mechanical disruption by the microdialysis catheter. In addition, this finding also suggests that at least some of the increase in plasma P1CP reported by Langberg *et al.* (1999) at 72 h post-exercise may have been due to tissue trauma.

In addition to tendinous tissue, type 1 collagen is also up-regulated in skeletal muscle, both in the endomysial and perimysial spaces (at 2 days post-exercise) and the interstitial space (at 8 days) following eccentric loading (Crameri *et al.*, 2004). Importantly, this appears to be the first study to have measured P1NP as a marker of type 1 collagen formation although, as the serum levels of P1NP were not measured, it was not clear if P1NP was concomitantly increased in the circulation or, like P1CP, remained unchanged. Subsequent studies using the microdialysis technique also report varying results regarding the effect of acute exercise on tissue levels of P1NP. Miller *et al.* (2005) observed no change in muscle P1NP and a 23% reduction in tendon P1NP 72 h after 60 min of one-legged knee extensor exercise at 67% workload max (W_{max}) in men, while later, following the same exercise protocol, an increase in tendon P1NP in women is reported (Miller *et al.*, 2007; Hansen *et al.*, 2008). In premenopausal women this increase is independent of menstrual cycle phase (Miller *et al.*, 2007) but attenuated in oral contraceptive users (Hansen *et al.*, 2008), while no increase is seen in postmenopausal women with and without estradiol replacement therapy (Hansen *et al.*, 2009).

Despite these increases in P1NP in the interstitial fluid surrounding both tendon and muscle tissue, the only study to measure serum P1NP found no change in its levels at 6h, 24 h, 48 h and 72 h following exercise (Miller *et al.*, 2005). Taken together, these results point to the upregulation of type 1 collagen synthesis in muscle and tendon by exercise but that this response is not reflected in the serum concentration of P1NP. Thus, as in rested humans, in the hours and days following exercise, the absolute and relative contribution of non-bone sources to circulating P1NP appears to remain unchanged suggesting P1NP levels continue to mainly reflect the synthesis of type 1 collagen in bone.

2.2.2.2 Osteocalcin

Osteocalcin (OC) is the major non-collagen protein in the bone matrix and is produced exclusively by osteoblasts, odontoblasts and hypertrophic chondrocytes (Boivin *et al.*, 1990; Weinreb *et al.*, 1990). Its structure contains three gammacarboxyglutamic residues that are γ-carboxylated by vitamin K, converting the glutamic acid residues to γ-carboxyglutamic acid (Gla) (Hauschka *et al.*, 1989). γ-carboxylation and the creation of Gla residues provide osteocalcin with the ability to bind bone hydroxyapatite with a high affinity (Dowd *et al.*, 2003; Hoang *et al.*, 2003). Thus, OC is thought to function as a localization site for hydroxyapatite crystals during bone matrix synthesis and play a role in the organisation of the extracellular matrix. Via Gla residues, about 60 to 90% of newly synthesized osteocalcin is absorbed into the bone hydroxyapatite, with the remainder leaking into the circulation where levels of immunoreactive OC correlate well with the bone formation rate as assessed by histomorphometry (Delmas *et al.*, 1985).

Although osteoblasts synthesize only intact OC, following secretion, OC is also degraded resulting in both intact peptides and smaller fragments in the circulation (Taylor *et al.*, 1990; Baumgrass *et al.*, 1997). The heterogeneity of OC fragments has been a substantial limitation in the clinical application of the serum OC assay (Delmas *et al.*, 1990) with different immunoassays producing varying results (Diaz Diego *et al.*, 1994).

Although previously thought to be involved only in the process of osteoid mineralisation, rather than have impaired mineralisation, OC knockout mice have increased cortical and trabecular thickness (Ducy *et al.*, 1996) suggesting the precise function of OC is still to be fully understood. As most OC is incorporated into the bone matrix it is conceivable that OC fragments may be released even during bone resorption. Indeed, osteoclastic bone resorption *in vitro* has been shown to release immunoreactive OC, both intact and fragmented molecules (Salo *et al.*, 1997; Ivaska *et al.*, 2004).

Recently, assays have been developed for OC fragments in urine (Ivaska *et al.*, 2005). Intact, unfragmented OC is yet to be been found in urine but includes a heterogeneous pool of OC fragments that consist mainly of the middle portion of the molecule. High concentrations of these fragments in urine are associated with low bone mass, and levels correlate with bone turnover rate assessed by conventional serum markers (Ivaska *et al.*, 2005). Different kinetics have been observed for serum and urinary OC. For example, in response to alendronate therapy, changes in u-OC but not s-OC correlate with established markers of bone resorption (Srivastava *et al.*, 2002), suggesting that urinary OC potentially offers an alternative method to monitor bone resorption. Due to the recovery and degradation of OC, however, urinary OC levels might better reflect basal bone turnover rather than acute changes in bone metabolism (Ivaska *et al.*, 2005).

Osteocalcin has also been identified as a bone-derived hormone that regulates energy metabolism. One study showed that treatment of patients with an anticoagulant that decreases carboxylation of osteocalcin, result in hypoglycemia (Hetzel *et al.*, 2006), while homozygous osteocalcin-gene deficient mice develop a phenotype that appears to be very similar to type 2 diabetes (Lee *et al.*, 2007). Additionally, *in vivo* treatment of healthy mice with non-γ-carboxylated OC was associated with increased pancreatic b-cell numbers, insulin secretion, energy expenditure and insulin sensitivity (Ferron *et al.*, 2008). Combined with observations from other mouse models, these results have identified OC as a bone-derived hormone that, in its undercarboxylated form, regulates glucose metabolism indicating that OC is the only molecule made by osteoblasts that can act in this way (Lee and Karsenty, 2008). In humans, serum OC is inversely related to fat mass and plasma glucose in men (Kindblom *et al.*, 2009) and lower in postmenopausal women with type 2 diabetes than in controls (Im *et al.*, 2008). Both these studies used assays that measured total OC but the same associations with undercarboxylated OC were subsequently confirmed by Kanazawa *et al.* (2010).

Acting in a hormone-like manner, OC may also explain the apparent association between the use of certain anti-depressant drugs, negative changes in bone and disturbances in glucose homeostasis (Derijks *et al.*, 2008) via a pathway involving serotonin (5-HT) and the low-density lipoprotein receptor-related protein (Lrp5). 5-HT is generated in both brainstem neurons and enterochromaffin cells of the duodenum, and both centrally and peripherally, the duration and intensity of serotonergic activity is regulated by the 5-HT transporter (5-HTT). Functional receptors for 5-HT exist in primary and clonal osteoblasts, osteocytes and periosteal fibroblasts, a population containing osteoblast precursor cells (Bliziotes *et al.*, 2001; Westbroek *et al.*, 2001; Bliziotes *et al.*, 2002). Osteoblasts, osteoclasts and osteocytes all possess the 5-HTT (Bliziotes *et al.*, 2001; Bliziotes *et al.*, 2002; Battaglino *et al.*, 2004) which is functional and highly specific for 5-HT uptake in osteoblastic and osteocytic cells (Bliziotes *et al.*, 2001, 2002). There is evidence that chronic use of selective 5-HT reuptake inhibitors (SSRIs) can result in reduced bone mass (Richards *et al.*, 2007), increased postmenopausal bone loss (Diem *et al.*, 2007) and fracture risk (Haney *et al.*, 2010). In mice at least, this negative change in bone quality appears to result from a reduction in bone formation, reflected in a ~50% fall in OC concentrations (Bonnet *et al.*, 2007).

Gut-derived serotonin (GDS) accounts for 95% of total serotonin and is synthesised via a pathway involving the rate-limiting enzyme tryptophan hydroxylase 1 (Tph1) that is preferentially expressed in the periphery. Although most GDS is taken up by platelets, a small fraction remains in the circulation leaving open the possibility of a hormonal action. Compared to wild-type, in the bones of $Lrp5^{-/-}$ mice, the gene most highly expressed is *Tph1* while it is also overly expressed in the duodenum and serum 5-HT levels are also elevated (Yadav *et al.*, 2008). These mice also exhibited decreased expression of genes associated with cell proliferation, but not osteoblast differentiation, bone matrix deposition, or osteoclast differentiation, but have a paucity of osteoblasts despite normal *ex vivo* osteoblast differentiation. In these mice a low tryptophan diet normalised serum 5-HT levels as well as the skeletal phenotype and further studies of transgenic mice with either gut- or osteoblast-specific loss- or gain-of-function of Lrp5 support Lrp5 as regulating bone formation indirectly via effects in the gut and confirm 5-HT as the intermediary.

However, the negative skeletal effects of elevated circulating 5-HT also do not explain the negative skeletal effects of SSRIs on bone formation as chronic administration of the same SSRI over 1-2 weeks results in reduced serum 5-HT levels (Rothman *et al.*, 2008; Zolkowska *et al.*, 2008). Interestingly, chronic suppression of 5-HT through dietary-denial of tryptophan results in growth retardation, impairment of normal bone acquisition of the appendicular skeleton, lower (> 50%) levels of osteocalcin and a marked drop in insulin growth factor (IGF)-1 (Sibilia *et al.*, 2009) suggesting that some degree of 5-HT signalling is important in normal bone formation. SSRIs might impact the skeleton by directly inhibiting the 5-HTT located on bone cell membranes, increasing local 5-HT

levels, in spite of decreased circulating 5-HT, by reducing its removal from the bone cell microenvironment (Warden *et al.*, 2010).

Patients with osteoporosis–pseudoglioma syndrome (OPPG) a condition thought to result from a mutation in the Lrp5 gene have severe osteoporosis and elevated circulating 5-HT levels (Yadav *et al.*, 2008). Mice with targeted disruption of Lrp5 have low bone mass (Kato *et al.*, 2002) and, on a normal diet, show impaired glucose tolerance and glucose-induced insulin secretion (Fujino *et al.*, 2003) while a mutation in the LRP5 homolog LRP6 was found in a family with autosomal dominant early onset coronary artery disease, metabolic syndrome, and osteoporosis (Mani *et al.*, 2007). More recently, Saarinen *et al.* (2009) report a high prevalence of osteoporosis and abnormal glucose metabolism in 13 subjects with Lrp5 mutation and a tendency for higher serum 5-HT levels. As OC released from bone is thought to play a role in the regulation of glucose metabolism, it is possible that in patients taking SSRIs, the increased prevalence of disturbances to glucose homeostasis (Derijks *et al.*, 2008) might be related decreased serum OC levels resulting from the disruption of normal 5-HT signalling and the associated suppression of bone formation.

2.2.2.3 Alkaline phosphatase and bone specific alkaline phosphatase

Alkaline phosphatase is a ubiquitous enzyme present in most cells at, or near, the membrane (Moss, 1982), and is encoded by four human genes that encode for placental, germ cell, intestinal and a single tissue non-specific isoenzyme form (Seargeant and Stinson, 1979). The tissue non-specific form is expressed in tissues including the kidney, liver, and bone, with 95% of the total ALP found in the circulation originating from either the liver or bone. Its ubiquitous expression means total ALP is considered a non-specific marker of bone activity.

The bone isoform of ALP (bone ALP) is produced by osteoblasts and is released in an anchor-intact form, attached to the cell membrane by a hydrophobic glycosylphosphatidylinositol (GPI) anchor. Bone ALP is thought to catalyse the hydrolysis of PO_4 esters at the cell surface and, in doing so, provide a high PO_4 concentration essential for calcification of the skeleton, mineralization and bone formation (Fishman, 1990). Its activity is reported to be necessary for the initiation of mineralisation but not for the continuation of the process (Tenenbaum and Palangio, 1987; Bellows *et al.*, 1991; Barling *et al.*, 1999; Wennberg *et al.*, 2000). In adults with normal liver function, approximately 50% of the total AP activity in serum arises from bone with the remaining 50% derived from the liver (Green *et al.*, 1971). In children and adolescents, the bone-specific isoenzyme predominates – up to 90% – because of skeletal growth (Van Hoof *et al.*, 1990; Magnusson *et al.*, 1999).

That bone ALP is a more specific marker of bone formation than total ALP is supported by several lines of evidence. Compared with total ALP, bone ALP levels correlates better with PTH and height velocity (Nailk *et al.*, 1977) while bone ALP, but not total ALP shows a significant seasonal variation concomitantly with other specific markers of bone turnover (Woitge *et al.*, 2000). Bone ALP

concentrations show a greater increase that total ALP following menopause (Gonnelli *et al.*, 1996; Takahashi *et al.*, 1997) and are more sensitive than total ALP in patients with osteoporosis, primary hyperparathyroldism, hyperthyroidism, acromegaly, bone metastasis and hemodialysis patients (Duda *et al.*, 1988; Garnero and Delmas, 1993; Gonnelli *et al.*, 1996; Urena *et al.*, 1996). Bone ALP also displays a greater response to antiresorptive therapy (Garnero and Delmas, 1993; Watts *et al.*, 2001). Taken together, these findings point to bone ALP being preferable to total ALP as a specific marker of bone formation.

Early methods for the quantification of bone ALP included heat inactivation (Moss and Whitby, 1975), agarose gel electrophoresis (Van Hoof *et al.*, 1988), and wheat germ lectin precipitation (Rosalki and Foo, 1984). These methods were superseded by a two-site immunoradiometric assay (Tandem-R Ostase) which detected the bound isoform with a labelled second monoclonal antibody and showed good agreement with the electrophoresis method in Paget's patients (Panigrahi *et al.*, 1994). Changes in bone ALP concentrations using this assay are reported following menopause and metabolic bone diseases including primary renal osteodystrophy and hyperparathyroidism, with a cross-reactivity of 11-16% (Garnero and Delmas, 1993). Gomez *et al.* (1995) developed an enzyme immunoassay measures the activity captured, in both cases, to a solid-phase-bound monoclonal antibody. This assay has a 3-8% cross-reactivity and measured increases in bone ALP in patients with osteoporosis, Paget's disease, osteomalacia and primary HPT, and produced strong correlations with the immunometric assay and wheat germ method (Gomez *et al.*, 1995) as well as the electrophoresis method (Hata *et al.*, 1996).

Using the assay of Gomez *et al.* (1995), Withhold *et al.* (1996) detected a more pronounced increase in bone ALP than total ALP in patients with renal transplants and the decrease in osteoblast activity in multiple myeloma, and was as good as total ALP at detecting bone metastases in tumour patients. Increases in bone ALP are also reported following menopause using this assay (Hata *et al.*, 1996). Several studies have compared the performance of the two methods. Price *et al.* (1995) report a significant difference in the relation between activity captured by the monoclonal antibody and that found by the electrophoretic method in samples from children and Paget's patients, while no difference existed with the electrophoretic method. This finding was subsequently confirmed in children, Paget's patients and patients with obstructive liver disease (Price *et al.*, 1997) and might result from the immunocapture method recognising an isoform of bone ALP that is different from that recognised by the immunometric assay. Both assays have a similar cross-reactivity for the liver isoform (Price *et al.*, 1997).

More recently, Broyles *et al.* (1999) describe an enhanced version of the Ostase enzyme immunoassay (Tandem-MP Ostase) with a similar cross-reactivity but an improved assay imprecision and lower detection limit. This assay measured increases in serum bone ALP following menopause and a decrease following bisphosphonate treatment of Paget's patients. Comparing this assay with the

Tandem-R assay, Broyles *et al.* (1999) report similar reference intervals in healthy adults and bone assays detected increases in postmenopausal women compared with premenopausal women and decreases in Paget's patients following antiresorptive treatment. Magnusson *et al.* (2001) compared both Ostase assays with the immunocapture assay and found no difference in bone ALP levels in patients suffering from chronic renal failure.

Unlike other bone formation markers that are not associated with the fracture risk, bone ALP does predict fragility fractures in some cohorts (Garnero *et al.*, 2000; Ross *et al.*, 2000; Sornay-Rendu *et al.*, 2005). Bone ALP may be useful in predicting the response to pharmacological interventions. The early decrease in the bone ALP levels is associated with the long-term anti-spinal fracture efficacy in patients treated with raloxifene (Bjarnason *et al.*, 2001; Reginster *et al.*, 2004). Bauer *et al.* (2004) report that a greater reduction in bone ALP is associated with fewer osteoporotic fractures in alendronate-treated women, with each standard deviation reduction in the change in bone ALP at one year associated with fewer spine, non-spine and hip fractures. Those with a $\geq$30% reduction in bone ALP had a lower risk of non-spine and hip fractures relative to those with reductions of less than 30%. In patients with metastatic prostate cancer treated with zoledronic acid, the reduction in bone ALP levels correlates with improvements in the Visual Analogue Scale for pain (Fulfaro *et al.*, 2005).

In response to PTH therapy, as with the other bone formation markers, bone ALP is significantly increased after only 3 days, although the relative increase from pre-treatment is much less (Glover *et al.*, 2009) and remains so up to 28 days (Chen *et al.*, 2005; Dobnig *et al.*, 2005; Glover *et al.*, 2009). Unlike other bone formation markers that peak after 1 month of therapy, from 1 month onwards, bone ALP concentrations continue to increase up to 12 months (Chen *et al.*, 2005; Dobnig *et al.*, 2005). The one month change in bone ALP with PTH therapy is associated with improvements in indices of trabecular architecture at 22 months (Dobnig *et al.*, 2005).

In patients with Type II diabetes, Kanazawa *et al.* (2009) report that the OC to bone ALP ratio significantly correlated with vertebral fractures independent of BMD. As Bone ALP is expressed in the early period of osteoblast differentiation and OC in the latter, it has been suggested that bone ALP may be used in conjunction with OC as a measure of osteoblast maturation and that the OC to bone ALP ratio may help to compensate for the ineffectiveness of BMD to evaluate fracture risk in type 2 diabetes (Kanazawa *et al.*, 2009).

Despite ALP expressed in the liver and bone sharing the same primary amino acid sequence, it is possible to further differentiate this isoenzyme form into at least three liver isoforms, L1, L2 and L3, and three bone isoforms, B1, B2 and the minor fraction bone/intestinal (B/I) (Magnusson *et al.*, 1992; Magnusson *et al.*, 1993). The relatively abundance of the different isoforms of bone ALP varies with bone compartment. In cortical bone B1 activity is ~2-fold higher than B2 (~40% vs ~20% of total ALP content), whereas in trabecular bone, the opposite is true (Magnusson *et al.*, 1999; Magnusson *et*

al., 2002; Magnusson *et al.*, 2010). In both compartments, B/I represents ~15-20%, with a novel isoform, B1x (see below) accounting for the remaining ALP content (Magnusson *et al.*, 2002; Magnusson *et al.*, 2010). In cortical bone with severe Pagetic involvement, however, there is an altered profile, with relatively less B1, and a greater proportion of B/I and B1x (Magnusson *et al.*, 2010). In severely involved trabecular bone, the relative proportion of B2 is reduced while B/I and B1x are increased.

In the serum of healthy adults, the B1 and B2 isoforms account for the majority of the bone ALP content, reflecting the release of bone ALP from mineralised tissues. However, the serum levels of B1 and B2 isoforms can vary independently. During the growth spurt, girls aged 15-16 y and in the pubertal stage groups IV-V, reached a higher B1 to B2 ratio due to a more rapid decline of B2 compared with B1 (Magnusson *et al.*, 1995). B1 but not B2 levels are reduced after 1 week of IGF-1 and 1 month of GH therapy although both were increased after 3 months of GH (Magnusson *et al.*, 1997). In patients with skeletal metastases, all bone isoforms were increased compared with healthy controls, with B/I increased by ~100%, B1 by ~4-fold and B2 by ~10-fold (Magnusson *et al.*, 1998). Additionally, the relative contribution of B2 to total ALP levels was than twice that in Paget's compared with healthy controls (75% *vs* 35%) whereas it was unchanged for B/I and B1. In adult patients with chronic renal failure, both B/I and B2 are elevated compared with healthy controls, whilst B1 levels were not different (Magnusson *et al.*, 1999; Magnusson *et al.*, 2001). Like in adults, in child patients, 55% have B/I levels above the reference range although both B1 and B2 tend to be normal (Swolin-Eide *et al.*, 2006).

In Paget's patients stratified into high and low total ALP activity groups, high activity was associated with higher levels of B1, B2 and B/I compared with low activity and healthy controls (Magnusson *et al.*, 2010). The contribution of B2 to total ALP activity in high was ~100% higher than in control and 40% higher than the low activity group. In the low group, only B2 was increased compared with control suggesting that B2 might be a useful marker in when ALP activity is low such as in monostotic Paget's. Although overall, the significance of these differential changes in isoforms B1 and B2 have yet to be fully determined but some authors have suggested that they might reflect different stages of osteoblast maturation (Magnusson *et al.*, 1998).

There are distinctive differences between the profile of bone ALP isoforms in bone and serum. Despite accounting for >30% of total ALP content in bone, in serum, B/I represents only ~5% of total activity (Magnusson *et al.*, 2002). B/I is not a pure bone ALP isoform, as it co-elutes with intestinal ALP and is composed of approximately 70% bone and 30% intestinal ALP. B/I levels are significantly elevated in both adults and children with kidney disease (Magnusson *et al.*, 2001; Swolin-Eide *et al.*, 2006) although the significance of this increase remains to be fully determined.

In serum, bone ALP is in an anchorless form indicating the conversion of the anchor-intact form. This conversion is thought to occur through the enzymatic activity of GPI-specific phospholipase D (GPI-PLD), which circulates in much higher concentrations than the C form (GPI-PLC). However, GPI-PLD activity is not active toward GPI-anchored proteins on the surface of intact cells, suggesting bone ALP might initially be released from osteoblasts in an anchor-intact form, possibly associated with membrane vesicles, which are, only then, susceptible to GPI-PLD in the circulation (Low and Huang, 1991). Bone ALP isoforms have different patterns of glycosylation, with B1 and B2 having more sialic acid residues compared with B/I (Magnusson *et al.*, 2002). As the content of sialic acid in Bone ALP, liver ALP, intestinal, and placental ALP, determines their various clearance rates and corresponding biological half-lives (Clubb *et al.*, 1965; Komoda and Sakagishi, 1978), it was hypothesised that the difference in B/I content in bone and serum (*i.e.* the relatively low serum content) may be due to differences in post-translational glycosylation patterns of the Bone ALP isoforms, with a lesser number of sialic acid residues resulting in more efficient clearance and a shorter half life (Magnusson *et al.*, 2002).

More recently, using weak anion-exchange HPLC with post-column reaction detection, a fourth isoform, termed B1x has been described, located between B/I and B1 in the chromatographic profile (Magnusson *et al.*, 1999). B1x was first identified in extracts of bone from adult patients with severe renal insufficiency (Magnusson *et al.*, 1999). Measurable concentrations of B1x has subsequently been confirmed in the serum of 60% of these patients on dialysis (Magnusson *et al.*, 2001) and 7% (n = 2) of children with kidney disease (Swolin-Eide *et al.*, 2006) but not in healthy subjects (Magnusson *et al.*, 2001). Both adults and children with kidney disease who have measurable levels of B1x have normal levels of B/I (Magnusson *et al.*, 2001; Swolin-Eide *et al.*, 2006). In the study of Swolin-Eide *et al.* (2006), one child with measurable levels of B1x subsequently underwent growth hormone therapy after which it was no longer detectable suggesting that B1x is not continuously released from bone. B1x is also present in the serum of 63% of adult patients with kidney disease prior to dialysis (Haarhaus *et al.*, 2009) but not in Paget's patients (Magnusson *et al.*, 2010) despite representing a major proportion of ALP in bone tissue extracts.

In one study of patients with kidney disease (Magnusson *et al.*, 2001), B1x was associated with low bone turnover and correlated with isoform B1 ($r = 0.53$), P1NP ($r = 0.55$) and PTH ($r = 0.49$) although in a subsequent study associations with low bone turnover and PTH were not present and it did not correlate with GFR (Haarhaus *et al.*, 2009). As B1x was associated with a higher serum PO_4 and a higher calcium x PO_4 product (Haarhaus *et al.*, 2009), and both high PO_4 and a high calcium x PO_4 product are associated with vascular calcification (Schoppet and Shanahan, 2008), it has been hypothesised that B1x could originate from ectopic tissue calcification (Haarhaus *et al.*, 2009). Like the B/I isoform, the low serum concentrations of B1x compared to its relatively high content in bone might be explained by its lesser sialylation and thus more efficient clearance and shorter half life.

2.3 General limitations of bone turnover markers and pre-analytical variability

The reliability of the interpretation of biological measurements is increased if their pre-analytical and analytical variability is low. Inter- and intra-assay coefficients of variation are used to assess the degree of analytical variability and are dependent on the marker being examined and the method used. Pre-analytical variability comprises a range of factors that can be broadly divided into two groups: those over which the investigator can exert a high level of control, and those that are much less easily modified. Although, as with all biological measurements, analytical variation is vital to interpretation, in the case of BTM, the large number of factors that can affect their concentrations, and the relative potency of these effects, makes pre-analytical variability also of considerable importance.

2.3.1 Highly controllable factors

2.3.1.1 Circadian rhythm and pre-sample nutritional status

Some, but not all BTM, display a pronounced and significant circadian rhythm. Concentrations of all bone resorption markers peak in the early morning between approximately 0300 h and 0700 h and show a rapid and pronounced fall in the late morning, reaching a nadir in the late afternoon (Nielsen *et al.*, 1990; Pietschmann *et al.*, 1990; Eastell *et al.*, 1992; Bollen *et al.*, 1995; Aoshima *et al.*, 1998; Gertz *et al.*, 1998; Wichers *et al.*, 1999; Qvist *et al.*, 2002). This rhythm is independent of age, menopausal status, the 24 h cycle of changes in posture, activity, light and cortisol production (Qvist *et al.*, 2002). Despite the good agreement between different markers of bone resorption, there is considerable variation in the amplitude of the rhythm with serum and urinary β-CTX fluctuating up to 40% to 60% of the average concentration compared to only 10% to 35% for DPD and serum or urinary NTX. In contrast, serum TRACP 5b levels exhibit a modest circadian variation amplitude of only 14% (Hannon *et al.*, 2004) consistent with the lack of a significant change in number of osteoclasts during a 24 h cycle as demonstrated by histomorphometry (Simmons *et al.*, 1988).

A circadian variation is also observed in bone formation markers including OC (Nielsen *et al.*, 1990; Pietschmann *et al.*, 1990; Eastell *et al.*, 1992) and P1CP (Hassager *et al.*, 1992; Pederson *et al.*, 1995) and P1NP (Ahmad *et al.*, 2003). The amplitude of this rhythm, however, is considerably less (~5 to 25%). It has become apparent that the intake of food is an important pre-analytical modifier of the circadian rhythm of BTMs being most evident for plasma β-CTX. Fasting reduces the morning decrease in the plasma β-CTX (Christgau, 2000) while the variation about the 24 h mean is reduced from 35-40% to around 15% (Schlemmer and Hassager, 1999; Christgau, 2000; Qvist *et al.*, 2002). Subsequent studies showed that the ingestion of a mixed meal (Clowes *et al.*, 2002a), glucose (Bjarnason *et al.*, 2002; Clowes *et al.*, 2003; Henriksen *et al.*, 2003), protein (Bjarnason *et al.*, 2002; Henriksen *et al.*, 2003), fat (Bjarnason *et al.*, 2002; Henriksen *et al.*, 2003) and calcium (Horowitz *et al.*, 1994; Guillement *et al.*, 2000; Sadideen and Swaminathan, 2004) are associated with lower levels of BTM (Table 2.3).

Similarly, as with their circadian rhythm, bone formation markers show a considerably smaller response, with P1NP being reduced by 4-8% (Clowes *et al.*, 2002a; Clowes *et al.*, 2003) and, in the case of OC, ranging from a 16% reduction by a mixed meal (Clowes *et al.*, 2002a) (Table 2.3) to no effect of individual macronutrients (Henriksen *et al.*, 2003) (Figure 2.3). Bone ALP is not affected by acute food intake (Clowes *et al.*, 2002a) (Table 2.3). In time-course studies, the ingesting of macronutrients in the morning produces a day time variation in BTM similar to that seen in free-feeding individuals (Bjarnason *et al.*, 2002; Henriksen *et al.*, 2003) (Figure 2.3). As with the circadian rhythm of BTM, plasma β-CTX is the mostly strongly influenced, being suppressed by approximately 50% (Figure 2.4) while TRACP 5b is relatively unaffected (Hannon *et al.*, 2004).

Table 2.3. Percentage difference in bone turnover markers following a meal compared with fasting. *Adapted from Clowes et al. (2002a) and Hannon et al. (2004).*

Bone marker	Difference (fed - fasting) ± SE (%)
Bone ALP	-1.6 ± 1.0
OC	-4.1 ± 1.1[b]
P1NP	-3.8 ± 0.9[c]
u-fDPD	-7.5 ± 1.5[c]
uNTX	-7.9 ± 3.7[a]
uβ-CTX	-7.0 ± 2.6[b]
Plasma NTX	-8.5 ± 1.7[c]
Plasma β-CTX	-17.8 ± 2.6[c]
TRACP 5b	-2.4 ± 0.79[a]

[a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$ (paired t-test).

The suppression of bone resorption markers is maximally expressed 2 to 3 h after ingestion of macronutrients and resolved by 4 to 5 h (Bjarnason *et al.*, 2002; Henriksen *et al.*, 2003) (Figure 2.4). The suppression of plasma β-CTX by calcium might last up to 10 h when ingested late at night (Sadideen and Swaminathan, 2004). As both circadian variation and food intake modify the circulating concentrations of BTM, it is currently recommended that, for their clinical interpretation, blood samples should be collected in the morning and in the fasted state (Szulc and Delmas, 2008).

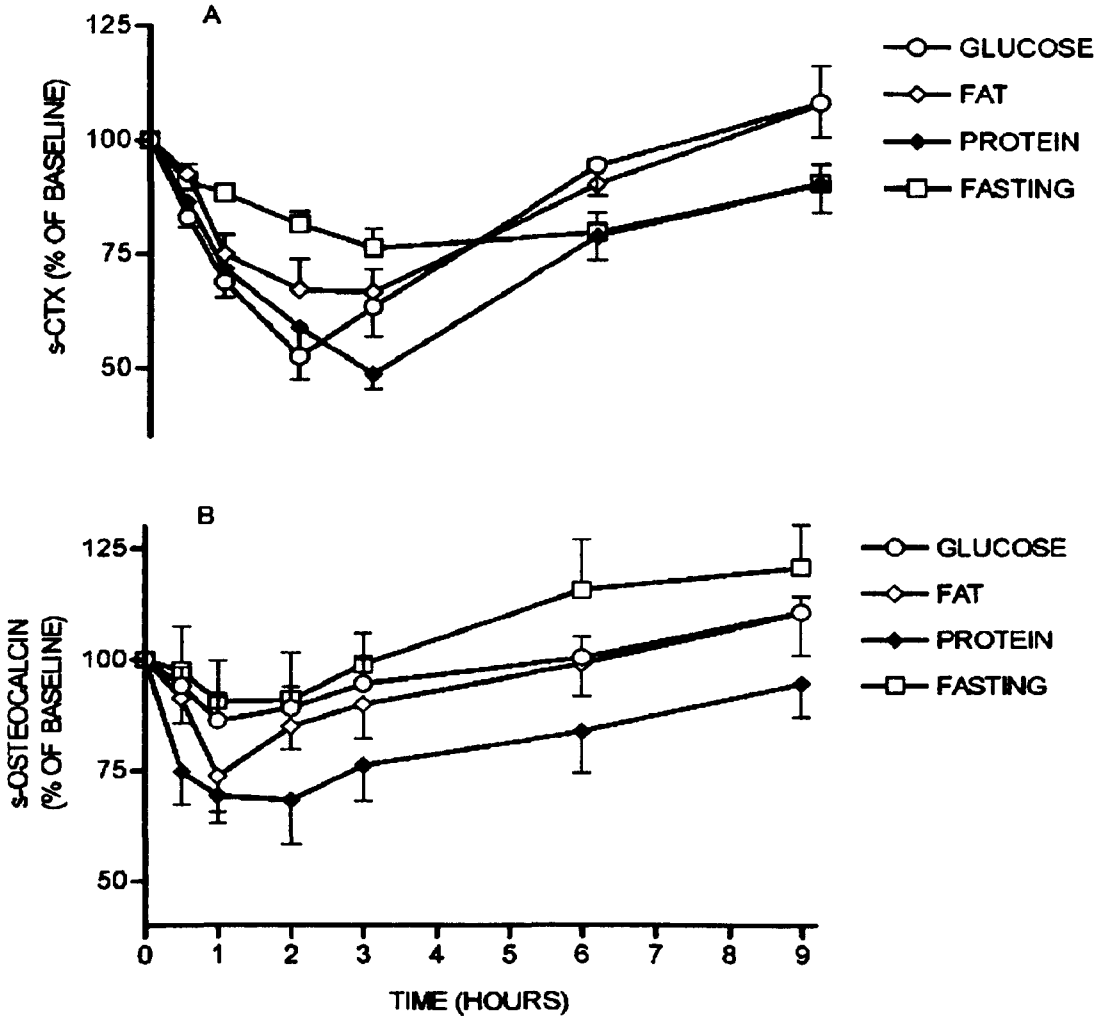

Figure 2.4. Effects of oral glucose, protein, and long-chain triglycerides on bone turnover in healthy individuals. The figure shows results of treatment with glucose, protein, long-chain triglycerides, and fasting control expressed as percentage of baseline for β-CTX (Panel A) and osteocalcin (Panel B) in healthy men and premenopausal women. Data are presented as mean ± SE. ANOVA (all) and p values for the individual groups compared with fasting controls are shown: *P < 0.05, **P < 0.01, ***P < 0.001. *Adapted from Henriksen et al. (2003).*

2.3.1.2 Pre-analytical variability in urinary bone turnover markers

To measure urinary bone resorption markers, a range of collection methods have been used, including uncontrolled spot samples, a first morning void (FMV), a second morning void (SMV) and a 24 h collection. When selecting a collection method it is a trade-off between the practical reliability and biological precision. The 24 h collection is considered the 'gold standard' method and is representative of the integrated daily excretion. The method is, however, cumbersome to perform and impractical for routine use (*e.g.* in outpatient clinics) and in research where subjects are not continuously confined and supervised in a laboratory setting. By contrast, the uncontrolled spot sample is the most convenient method of collection but, by being uncontrolled, sometimes fails to take

into account the diurnal variation of bone resorption markers (Eastell *et al.*, 1992) and the effects of feeding (Henriksen *et al.*, 2003). A FMV and SMV may provide a better signal to noise because of the high excretion of crosslinks during the early morning and will increase the chance of collecting a fasted sample. They also provide some control for the diurnal rhythm and allow serial samples to be collected in a more repeatable manner. From a clinical point of view, a SMV may provide an advantage over a FMV by coinciding more closely with an outpatient visit although the timing, and so the influence of the diurnal rhythm, of a 'freely supplied' SMV will likely vary considerably more than that of a FMV. Most of the reference data on urinary bone resorption markers were obtained from SMV samples. The correlation between SMV and 24 h collections is significant (r = 0.28-0.72), but varies according to the marker and the cohort investigated (Smith *et al.*, 2004).

In comparing the FMV, SMV, and 24 h collection methods, Leino *et al.* (1996) report concentrations of DPD to be ~17% higher in the FMV and SMV compared with the 24 h collection, with no significant difference between the FMV and SMV. This difference is explained by the diurnal variation in urinary DPD that results in higher concentrations at night that would be reflected in early morning spot samples. In contrast, the concentration of Hyp was unaffected by collection method. In terms of intra-individual variability, a SMV tended to produce a lower variability that the 24 h collection, which was in turn less variable than a FMV.

Fink *et al.* (2000) report no difference in mean concentrations or in iCV's for plasma β-CTX, NTX, fDPD and total DPD between FMV and SMV samples in nine, healthy pre-menopausal women when measured on four, non-consecutive days. Correlation coefficients between FMV and SMV samples for CTX, fDPD and total DPD were, however, only 0.46, 0.31, 0.68 respectively, and 0.64 for NTX which was not significant.

Smith *et al.* (2004) showed that correlation coefficients between the levels of NTX, PYD and DPD were higher in two, consecutive 24 h collections (R^2 = 0.52, 0.65, 0.76) than in two SMV (R^2 = 0.55, 0.26, 0.18) when expressed in nmol cross-link·nmol creatinine^{-1}, although all coefficients were significant (24 h collections: NTX, PYD and DPD (all $P < 0.001$); SMV: NTX, $P < 0.001$; PYD, $P < 0.001$; DPD, $P = 0.008$). There is a significant, but weak correlation in the levels of bone resorption makers corrected for creatinine between the SMV and the 24 h collection with R^2 values of 0.24, 0.33 and 0.08 for NTX, PYD and DPD (Smith *et al.*, 2004). These correlations remain significant although their strength is reduced slightly when the 24 h collection levels are expressed as nmol crosslink·day^{-1} to give total cross-link output (R^2 = 0.19, 0.19 and 0.08).

Uebelhart *et al.* (1991) report significant but weak correlation coefficients between levels of creatinine-corrected DPD and PYD (r = 0.30 and 0.29) in fasted single void urine samples and 24 h collections in postmenopausal women. The biological variation of free and total PYD and DPD did not differ significantly when compared in FMV samples (Abbiati *et al.*, 1993).

2.3.1.3 Stability of samples

Pyridinoline crosslinks in urine are sensitive to UV light (Sakura *et al.*, 1982; Casserly *et al.*, 1996), with a greater degradation seen for free than total pyridinolines (80% in fDPD *vs* 60% tDPD) after 3 days of exposure. No effect on urinary crosslinks was observed with laboratory light, either fluorescent lights and filtered daylight (Blumsohn *et al.*, 1995a; Walne *et al.*, 1995). A 1 cm layer of urine absorbs ~99% of all UV light and no effect of daylight is found on urine stored in a large (850 mL) container although in a small container, a small amount of degradation was observed (Sakura *et al.*, 1982). No degradation was observed at or below 20 °C (down to -70 °C) for up to 9 months (Gerrits *et al.*, 1995; Casserly *et al.*, 1996). Findings concerning freeze–thaw stability are inconsistent. The authors of one study (Gerrits *et al.*, 1995) found no degradation of crosslinks in urine after 10 freeze–thaw cycles, whereas the authors of another study found degradation after only five freeze-thaw cycles (Walne *et al.*, 1995).

2.3.1.4 Correction of urinary bone marker concentrations for kidney function and urine production

Urinary concentrations of bone resorption markers are frequently expressed as molar ratios with the urinary creatinine (Cr) concentration to correct for alterations in kidney function and thus urine concentration. The use of a biochemical correction factor automatically introduces additional variability from the analytical variability of the assay itself and, perhaps more importantly, from the biological variability in Cr excretion itself. Once formed, creatinine diffuses from cells and appears in the urine via glomerular filtration, and to a smaller extent tubular secretion. Folin's 'law' states that on a creatine-free diet the 24 h output of creatinine is constant from day-to-day for a given individual (Folin, 1904). Subsequently, however, several careful studies in healthy ambulatory subjects have challenged this assumption and shown a daily variation of 4-8% (Cryer and Sode, 1970; Greenblatt *et al.*, 1976).

Like bone resorption markers, the excretion of Cr also follows a 24 h rhythm, although the pattern of fluctuation is reversed, with the higher concentrations seen in the late afternoon (1630 h to 2030 h) and lowest in the early morning (0430 h to 0830 h) (Faucheux *et al.*, 1976). The degree of variation in Cr levels is affected by the sample collection method with the lowest intra-individual variability seen in 24 h collections (10-15%), which is increased in SMV (~30%) and increased further still in FMV (35-45%) (Shephard *et al.*, 1981; Gowans and Fraser, 1987; Howey *et al.*, 1987; James *et al.*, 1988; Bennett and Wilkins, 1993; Ricos *et al.*, 1994; Sebastian-Gambaro *et al.*, 1997). The inter-subject variability with these different methodologies shows a similar pattern and is of a comparable magnitude. The effect of this variation is reflected in the data of Smith *et al.* (2004), who showed that the correlation coefficient between Cr excretion ($mmol \cdot L^{-1}$) in two, consecutive SMV samples was low

($r = 0.27$), improved only slightly in 24 h samples ($r = 0.34$) and reached only 0.50 when expressed as $mmol \cdot day^{-1}$ in the same 24 h sample.

Despite the importance of Cr levels in the interpretation of changes in urinary bone markers, there is relatively little information regarding the effects of physical stress on urinary Cr levels. Physical stress, such as exercise is known to increase the urinary content of Cr. Srivastava *et al.* (1957) report a 9.4% increase in urinary Cr in a 24 h collection taken on the day of a 3 h march. Increasing the speed of the march (3.1 to 4.0 *vs* 2.9 to 3.0 $miles \cdot h^{-1}$) did not result in a further significant increase in Cr levels (+10.4%). Urinary Cr is increased 40% immediately after a 90 km ski race (Refsum *et al.*, 1973), while Refsum and Strömme, (1974) report a 17% increase in urinary Cr on the day of a 70 km skiing race compared with the day before the race. The day after the race, urinary Cr levels remained significantly higher than pre-race levels although the difference was only 5%. These increases were seen despite evidence of a decrease in Cr excretion (Refsum and Strömme, 1974).

Following an identical length ski race, urinary Cr is increased 45% immediately afterwards (Refsum and Strömme, 1975). With cycling exercise, Cr concentrations are unchanged for up to 48 h following 60 min of concentric and eccentric cycle ergometer at 70% and 40% VO_{2max} respectively (Plante and Houston, 1984) although they are increased after 2.5 h of cycling (De Palo *et al.*, 2003). Urinary Cr is increased 13% immediately after a bout of resistance exercise (3 sets of 10 repetitions at 70-75% 1-RM) (Timón Andrada *et al.*, 2007), while Turgut *et al.* (2003) report a 100% increase in urinary Cr immediately after 2 h of combined running and volleyball exercise. Finally, a 90% increase in Cr is reported immediately following a 160 km run (Nieman *et al.*, 2006) and 47% following a 100 km run (Décombaz *et al.*, 1979). In the later study, Cr levels remained 32% above pre-race levels 24 h after the race although this increase was no longer significant.

While the mechanism(s) of these exercise-induced changes in urinary creatinine output are unknown, it is known that creatinine results from the dehydration of creatine in the liver, kidney and muscle. As muscle accounts for 98% of the total body creatine pool, it is likely that the increase in urinary Cr with exercise reflects an increase in the turnover over of the creatine pool in muscle to support cellular energy metabolism and the related increase in the dehydration of creatine to creatinine (Heymesfield *et al.*, 1983). As severe trauma is also known to increase Cr excretion (Schiller *et al.*, 1979), it is also possible that some of this increase in urinary Cr levels may be due to exercise-induced muscle damage that can occur with strenuous, unaccustomed or eccentric muscle activity.

Despite this clear effect of exercise on urinary Cr concentrations, not all studies that have assessed changes in urinary bone markers, and used Cr as a corrective factor, have examined the effects of exercise on Cr concentrations. Brown *et al.* (1997) measured PYD and report no change in Cr for up to 9 days following a bout of 50 maximum effort, single leg, eccentric contractions of the knee

extensors, whereas Welsh *et al.* (1997), despite correcting PYD and DPD for Cr, do not report whether Cr levels changed following 30 min of walking. As with Refsum and Strömme, (1974), Ashizawa *et al.* (1997) report a decrease in Cr excretion rate measured at 1 h after a strenuous bout of resistance exercise but do not report actual urinary Cr levels. In contrast, no change in Cr excretion is seen in 24 h urine samples taken on the day of, and for 5 days after a strenuous bout of resistance exercise but, again actual urinary Cr levels were not given (Ashizawa *et al.*, 1997).

Like physical stress, psychological stress, such as during academic examinations, increases Cr although the increase is less pronounced (Scrimshaw *et al.*, 1966a). Accordingly, sleep deprivation for 24 h increases the coefficient of variation in creatinine excretion four-fold (Scrimshaw *et al.*, 1966b).

Changes in dietary protein, creatine and creatinine can also alter urinary Cr excretion. A chronic low creatine (vegetarian) diet is associated ~30% lower levels of urinary Cr (Delange *et al.*, 1989). Accordingly, Bleiler and Schedl, (1972) showed that the removal of creatine from a normal diet results in a progressive reduction in urinary Cr over an 8 to 11 week period. In this study, subjects consumed either 20 g, 70 g or 140 g of protein and increasing protein intake was associated with a smaller reduction in urinary Cr. Urinary Cr increased rapidly again with the resumption of a normal diet. In the short term, the acute ingestion of protein (beef or tuna) increases Cr clearance after only 1 h which remains elevated at 4 h and returns to baseline at 9 h following ingestion (Narita *et al.*, 1999).

In women, the phase of the menstrual cycle affects Cr excretion with higher values in the luteal phase, increased by ~20% from the week of menstruation (Davison and Noble, 1981; Bisdee *et al.*, 1989). During pregnancy, Cr levels are increased by 45% by the ninth week of gestation (Davison and Noble, 1981). In addition to these factors, a variety of endogenous and exogenous compounds, several drugs, age, infection, trauma, and renal insufficiency also increase Cr excretion. For a more extensive review of factors influencing Cr excretion see Heymsfield *et al.* (1983) and Vesper *et al.* (2002).

2.3.1.5 Effect of acute exercise and exercise training

Recent acute exercise may also influence BTM concentrations in subsequent samples (this data is reviewed in more detail in Section 2.6.1). P1CP is increased 96 h after a bout of intense concentric muscle activity (Virtanen *et al.*, 1993), and both P1CP and 1CTP are increased at 72 h after 45 min of light jogging (Thorsen *et al.*, 1997). Osteocalcin is reduced at 1 to 3 days in men and 1 to 5 days in women after a marathon with bone ALP reduced from 1 to 5 days in women but unchanged in men (Malm *et al.*, 1993). Langberg *et al.* (2000) report increased P1CP but not 1CTP 3 days after a marathon. Similar findings are reported after a 3 h run at 12 $km \cdot h^{-1}$ (Langberg *et al.*, 1999).

Findings from Herrmann *et al.* (2007) suggest that plasma β-CTX may be reduced by moderate exercise and increased 24 h after strenuous exercise lasting 60 min, although the authors do not report controlling for either the time of day or the nutritional state (*i.e.* fasted or fed) of their subjects prior to sample collection. Similarly, a decrease at 24 h is reported in P1NP and OC with moderate exercise,

although the stimulatory effect of strenuous exercise on bone formation markers at this time is less marked (Herrmann *et al.*, 2007). Neither plasma β-CTX nor P1NP is affected at 24 h after 30 min of brisk walking on a treadmill with and without a 5 kg load (Tosun *et al.*, 2006). Following ultra-endurance exercise, plasma β-CTX may remain elevated by 40% on the first morning post-exercise, although in this study the authors do not report standardising the nutritional status of subjects before blood sampling or tightly controlling the time of day that samples were taken (Kerschan-Schindl *et al.*, 2009).

In young adults, there is disagreement about the effect of long-term physical activity on BTM with previous studies reporting both higher (Nishiyama *et al.*, 1988; Hetland *et al.*, 1993) and more recently lower (Brahm *et al.*, 1997c) rates of bone turnover in those involved in regular physical activity compared with their less active counterparts. The onset of a physical training program is associated with changes in BTM, with aerobic training reducing bone resorption markers, and anerobic training increasing markers of resorption (total PYD and DPD) and formation (OC and bone ALP) after four weeks and eight weeks of training (Woitge *et al.*, 1998a). The onset of a resistance training program in a similar population is associated with an increase in OC and bone ALP at one month that is maintained at four months, whereas PYD remains unchanged (Fujimura *et al.*, 1997). In postmenopausal women, however, BTM levels are not significantly affected by a program of light physical exercise (Brooke-Wavell *et al.*, 2001) or daily physical activity (Suleiman *et al.*, 1997; Hia *et al.*, 2001).

2.3.1.6 Changes in energy availability

Much of the information on the relationship between energy availability and bone turnover comes from the study of conditions characterised by an eating disorder associated with reduced caloric intake. Eating disorders, particularly anorexia nervosa (AN) are associated with marked losses in body mass and BMD and present with pronounced changes in bone remodelling.

Grinspoon *et al.* (1996) report reduced concentrations of bone ALP accompanied by increased urinary DPD and NTX in AN patients. The increase in NTX was confirmed in a second study but was this time accompanied by unchanged concentrations of OC (Grinspoon *et al.*, 1999). In two different groups of AN patients, Stefanis *et al.* (1998) report increased DPD accompanied by normal levels of P1CP, and increased 1CTP with normal P1CP and OC. Zipfel *et al.* (2001) observed increased DPD but no change in OC while more recently, Soyka *et al.* (2002) observed significantly lower levels of DPD, NTX bone ALP and OC in AN patients compared with healthy controls. Heer *et al.* (2004) report no difference in serum β-CTX concentrations between AN patients and healthy controls but significantly lower levels of P1CP and bone ALP.

Weight loss in obese populations is associated with changes in both BMD and bone remodelling. Longer-term weight reduction (4 to 13%) in postmenopausal women leads to bone loss of approximately 1% and 4% compared with a weight-stable group (Svendsen *et al.*, 1993; Avenell *et al.*, 1994; Riedt *et al.*, 2005) and results in an increase in bone turnover, with greater increases in bone resorption than in formation (Ricci *et al.*, 1998; Ricci *et al.*, 2001; Shapses *et al.*, 2001). The effect of caloric restriction on bone turnover in obese populations may be confounded by the associated reduction in calcium intake. Indeed, in the studies mentioned above calcium intakes have been ~600 to 800 $mg \cdot d^{-1}$. When calcium intake is 1700 to 1800 $mg \cdot d^{-1}$, no acceleration in bone turnover is observed while 1000 $mg \cdot d^{-1}$ gives discordant results (Riedt *et al.*, 2005; Riedt *et al.*, 2007).

Much shorter periods of energy restriction can result in changes in bone turnover. Previous work has shown that a reduction in energy availability through exercise alone (*i.e.* increased energy expenditure) has no effect on BTM concentrations, but when energy availability is restricted by approximately 50%, a 15% reduction in P1NP occurs after only 3 days of exercise (Zanker and Swaine, 2000). This level of energy restriction had no effect on bone resorption markers. This finding was confirmed by Ihle and Loucks (2004), who, during a 5 day study, showed a dose response between energy available and bone formation marker levels. They also showed that when energy availability was severely restricted to only 10 $kcal \cdot kgLBM^{-1} \cdot day^{-1}$, markers of bone resorption were also increased, further disassociating resorption from formation.

Neither of these studies included a recovery phase, so following a short period of energy restriction, the time it takes for the restoration of BTM to their normal levels once energy availability is increased is unknown. With anorexia, however, where the duration of the restriction in energy availability is much longer, re-feeding results in a decrease in plasma β-CTX after three weeks and an increase in P1CP and bone ALP by seven weeks (Heer *et al.*, 2002) although it may take up to 15 weeks for bone formation markers to return to levels seen in healthy controls (Heer *et al.*, 2004).

2.3.2 Uncontrollable and less easily controlled factors

2.3.2.1 Season

Markers of bone turnover are higher in the winter compared with the summer (Chapuy *et al.*, 1996; Woitge *et al.*, 1998b; Meier *et al.*, 2004). This fluctuation might be related to the reciprocal change in PTH reported in some (Chapuy *et al.*, 1996; Meier *et al.*, 2004) although not all seasonal studies (Woigte *et al.*, 1998b) and, at least in part, to reduced vitamin D levels in the winter months (Chapuy *et al.*, 1996; Scharla *et al.*, 1996; Meier *et al.*, 2004).

2.3.2.2 Disease

Paget's disease and hyperparathyroidism may result in high bone turnover (Delmas *et al.*, 1986; Alvarez *et al.*, 1995) whereas hypoparathyroidism, hypopituitarism and adult growth hormone deficiency reduce levels of bone turnover markers (Mizunashi *et al.*, 1988; Colao *et al.*, 1999; Ahmad *et al.*, 2003). Cancers that result in bone metastases can also increased BTM levels (Leeming *et al.*, 2006; Voorzanger-Rousselot *et al.*, 2006). In rare instances, such as Cushing's disease and multiple myeloma, the disassociation of bone resorption from bone formation may occur (Alaxandrakis *et al.*, 2002; Kristo *et al.*, 2006).

2.3.2.3 Unloading and immobility

A disassociation between bone resorption and bone formation also occurs during periods of gravitational unloading such as during space travel and extended periods of bed rest, with bone resorption increased and bone formation either decreased or unchanged (Smith *et al.*, 1998; Smith *et al.*, 2005; Smith *et al.*, 2009). This effect is rapid, with the increase in bone resorption evident after only two days of unloading (Baecker *et al.*, 2003). Accordingly, some chronic diseases that result in forced, prolonged bed rest and/or limited mobility, increase levels of BTM, especially those of bone resorption (Sato *et al.*, 1998; Chen *et al.*, 2006).

2.3.2.4 Recent fracture

A recent fracture will also alter BTM levels (Yu-Yahiro *et al.*, 2001; Obrant *et al.*, 2005; Veitch *et al.*, 2006; Ivaska *et al.*, 2007; Stoffel *et al.*, 2007). Hip but not wrist fracture is associated with increased urinary OC in the hours following fracture, but no other markers are affected (Ivaska *et al.*, 2007). Bone formation markers are increased at three days post-fracture with bone ALP and OC but not P1CP increased further still at 60 days (Yu-Yahiro *et al.*, 2001). In patients who have undergone plate osteosynthesis, peak levels of resorption markers are seen before those of formation (TRACP 5b at ~10 to 20 days; 1CTP at 40 to 50 days; bone ALP, P1CP and OC at 80 to 90 days (Stoffel *et al.*, 2007). This data also suggests that fracture size may determine the BTM response with tibial fractures showing more marked increases than malleolar fractures and BTM remained increased even after bone union was complete. A similar trend – bone resorption markers increasing before bone formation markers – is also reported by Veitch *et al.* (2006), with plasma β-CTX increasing ~100% in 1 to 2 weeks, and P1NP and bone ALP at 12 weeks.

From 4 to 6 months post-fracture, markers of both resorption (u-DPD, TRACP 5b, plasma β-CTX) and formation (P1NP, bone ALP, OC) are elevated (Yu-Yahiro *et al.*, 2001; Ivaska *et al.*, 2007) with the most pronounced increases seen in hip factures (Ivaska *et al.*, 2007), although DPD may only be increased in patients with low BMD (Yu-Yahiro *et al.*, 2001). Beyond 6 months, concentrations of all BTM tend to fall but, with the exception of DPD, may remain elevated at 1 year post-fracture

(Yu-Yahiro *et al.*, 2001; Ivaska *et al.*, 2007) although in the study of Ivaska *et al.* (2007) the small changes in BTM at one year post-fracture might be confounded by the non-fasting state of subjects and the relatively wide time frame (0830 h to 1130 h) in which blood samples were collected. In the other study (Yu-Yahiro *et al.*, 2001) no description of sampling conditions is provided. Finally, one study suggests, by the author's visual estimation only, that BTM may remain elevated for 2 years after facture (hip, radial, vertebral) in elderly women (Obrant *et al.*, 2005).

2.4 The role of bone turnover in calcium metabolism

One of the skeletons many roles is to act as a large reservoir of readily available calcium. Tight regulation of the circulating ionised calcium (iCa) concentration ensures the optimum function of a range of physiological processes so access to the bone reservoir is vital. The secretion of PTH is key to accessing this reservoir and the circulating iCa concentration is a major regulator of PTH. PTH is secreted from the parathyroid glands, which contain on the surface of their chief cells, the calcium-sensing receptor (CaSR) that responds to changes in iCa (Tfelt-Hansen and Brown, 2005). Thus, a decrease in iCa results in an increase in PTH secretion and, following the restoration of calcium to within its normal range, a subsequent decrease in secretory activity. Similarly, the increase in iCa that occurs during periods of sustained, abnormally high PTH levels, such as in hyperparathyroidism or a PTH infusion, demonstrates PTH acting to control the calcium concentration and thus a bifunctional relationship between the two (Figure 2.5).

In addition to the stimulation of osteoclast activity in bone to release stored calcium, restoration of a normal circulating calcium concentration by PTH is achieved by two other mechansims: by increasing the tubular reabsorption of calcium in the kidney and by increasing the production of 1,25-dihyroxyvitamin D and thus calcium absorption via an increase in the activity of renal 1 hydroxylase in the bowel. On restoration of the circulating calcium concentration, PTH secretion decreases via a negative feedback loop via the calcium-sensing receptor. In conjunction with the actions of PTH, circulating calcium is maintained within normal range by the release of calcitonin by the thyroid that reduces the release of calcium and PO_4 from bone by inhibiting osteoclast activity and so bone resorption.

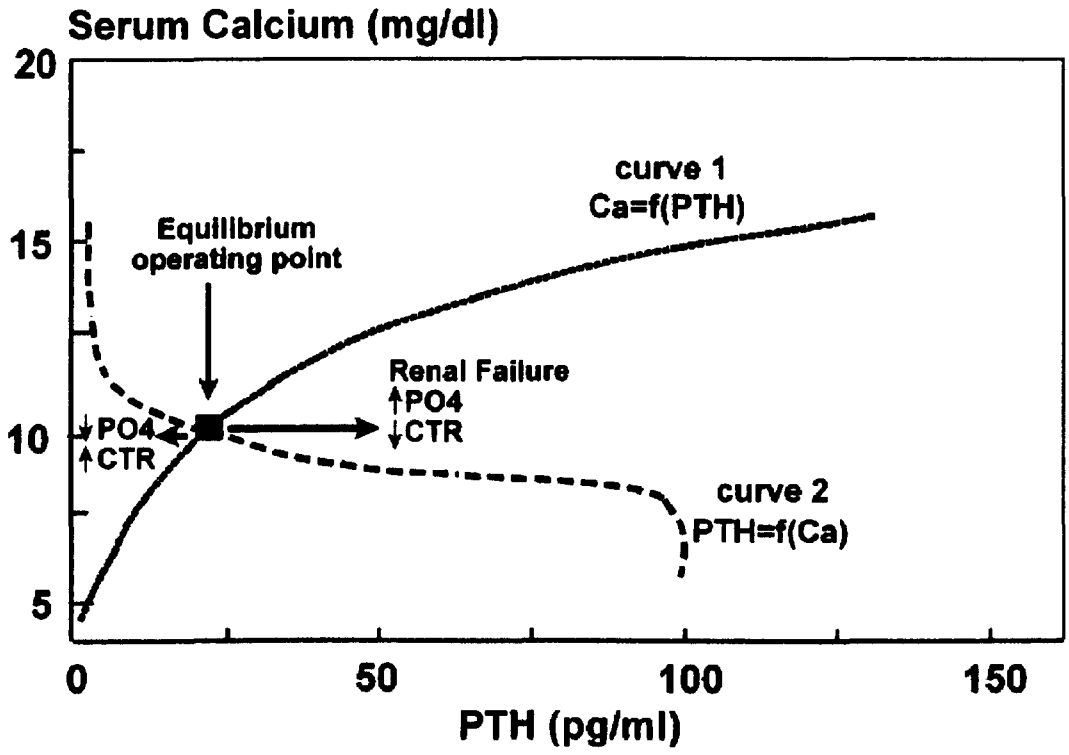

Figure 2.5 The representation of the relationship between serum calcium and parathyroid hormone (PTH) as that of a bifunctional one. Curve 1 shows serum calcium is a function of PTH and curve 2 that PTH is a function of serum calcium. The intersection of the two function curves determines the equilibrium operating point (EOP) for the serum calcium concentration. The effects of phosphate (PO_4) and calcitriol (CTR) and renal failure on EOP are also shown. *From Felsenfeld et al. (2007) adapted from Brent et al. (1988).*

The relationship between circulating calcium and PTH can be described by several parameters including 1) basal PTH – the PTH level without the induction of hypocalcemia or hypercalcaemia; *2*) maximal PTH – the highest PTH value observed in response to hypocalcemia and that an additional reduction of the serum calcium does not further increase PTH; 3) basal serum calcium – the serum calcium concentration at the basal PTH; *4*) the ratio of basal PTH to maximal PTH ratio; *5*) the set point of calcium – the serum calcium concentration at the mid-range of the PTH–calcium curve and; *6*) minimal PTH – the lowest PTH concentration measured during induction of hypercalcaemia which is never zero in healthy individuals (Felsenfeld *et al.*, 2007). The basal PTH value is reported to be around 25 $pg \cdot mL^{-1}$ with maximal PTH in the region of 100 $pg \cdot mL^{-1}$ while basal to maximal PTH ratio is approximately 20-30% (Ramirez *et al.*, 1993). This ratio is increased during hypocalcemia and decreases correspondingly during hypercalcaemia. The calcium set point is thought to reflect the sensitivity of the parathyroid gland, and thus PTH secretion, to the serum calcium concentration and indicates corresponding shifts in the PTH–calcium curve (Felsenfeld *et al.*, 2007).

The availability of a calcium 'clamp' technique has allowed the study of the calcium-PTH relationship under conditions of stable calcium concentrations (Fox and Heath III, 1981). Using this clamp, a 20% (from 9.6 to 7.6 $mg \cdot dl^{-1}$) reduction in circulating iCa is achieved within 1-2 min. This reduction increased PTH within 1 min which peaked at 4 to 10 min, increasing 5 to 17 fold from baseline. After 60 min, PTH levels had declined to 50% of peak levels despite constant hypocalcemia (Fox and Heath III, 1981). Similarly, acute restoration of calcium levels rapidly suppressed PTH to control levels. A longer (8 h) clamp, during which iCa was reduced by 1.7 $mg \cdot dL^{-1}$, induced a 5-fold increase in PTH by 15 min that remained increased 3.2-fold from baseline during the remaining 7 h (Fox and Heath III, 1981).

The calcium clamp in humans was first described by Tørring and Sjöberg, (1983a) to generate a controlled hypercalcaemia. An iCa level above the normal range (1.27 $mmol \cdot L^{-1}$) was reached within 10 min and 1.45 $mmol \cdot L^{-1}$ was obtained within 25 min and maintained in a steady state for 155 min. In this model, postmenopausal women showed a more rapid increase in iCa compared with premenopausal females and males, although no statistical difference between the three groups were observed in mean iCa levels during steady state hypercalcaemia or the iCa levels measured at 30 to 120 min of the infusion (Tørring and Sjöberg, 1983b). Calcium clamps in humans using either citrate or ethylenediaminetetraacetic acid (EDTA) that reduce iCa concentration by about 10-20% result in a rapid increase in PTH concentrations with the onset of the infusion (Brent *et al.*, 1988; Conlin *et al.*, 1989; Schwarz *et al.*, 1990; Schwarz *et al.*, 1992; Schwarz *et al.*, 1993; Schwarz *et al.*, 1994; Saha *et al.*, 1997; Schwarz *et al.*, 1998) (Table 2.4).

PTH concentrations peak at 5 to 15 min after the onset of hypocalcemia, with increases ranging from 2 to 20-fold. However, despite the continuous presence of hypocalcaemia in these studies, the peak in PTH is transient and subsequently concentrations decline but remain approximately 2 to 4-fold above baseline concentrations for the remainder of the infusion (Schwarz *et al.*, 1990; Schwarz *et al.*, 1992; Sørensen *et al.*, 1994; Saha *et al.*, 1997). The response of PTH to hypercalcaemia differs to that of hypocalcaemia. With the infusion of a range of calcium preparations, PTH is suppressed more gradually (and iCa increased) and there is no evidence of a transient nadir or partial recovery. Instead, PTH concentrations reach a steady state by about 30 to 50 min and remain there (Schwarz *et al.*, 1992; Ramirez *et al.*, 1993). The induction of hypocalcaemia (-0.2 $mmol \cdot L^{-1}$) and PTH release does not affect the suppressive effect on PTH of a subsequent bout of hypercalcaemia (+0.2 $mmol \cdot L^{-1}$) (Schwarz *et al.*, 1994).

Table 2.4. Effects of hypocalcaemia and hypercalcaemia on circulating parathyroid hormone (PTH) concentrations in humans.

Study	Method	iCa level ($mmol \cdot L^{-1}$)	Duration	Maximum PTH change (%change; level)	Time to max/min PTH	End PTH (% change, level)
Brent *et al.*	iv CG	↑ (NS)	60 min	↓72%; 6.4 $ng \cdot L^{-1}$	NS	NS
(1988)*	iv CG	↑ (NS)	120 min	↓76%; 5.6 $ng \cdot L^{-1}$	NS	NS
	iv EDTA	↓ (NS)	60 min	↑ NS; 95.0 $ng \cdot L^{-1}$	NS	NS
	iv EDTA	↓ (NS)	120 min	↑ NS; 99.9 $ng \cdot L^{-1}$	NS	NS
Conlin *et al.*	iv CIT	1.26-1.06 (↓15%)	NS	↑ 2.5-fold; 69.4 $ng \cdot L^{-1}$	NS	NS
(1989)*	iv C then iv CIT	1.42-1.26 +/- 0.02 (↓12%)	NS	↑ 7.1-fold; 55.0 $ng \cdot L^{-1}$	NS	NS
Grant *et al.*	iv CIT	1.27-10.6 (↓ 20%) in linear manner	120 min	(mean) ↑ 36.4 $pg \cdot mL^{-1}$	10 min	NS, ~60 $pg \cdot mL^{-1}$
(1990)*		1.27-10.6 (↓ 20%) with 4 x steps of ↓ 0.05	120 min (4 x 30 min step)	(mean) ↑ 19.4 $pg \cdot mL^{-1}$	10 min	NS, ~60 $pg \cdot mL^{-1}$
Schwarz *et al.*	iv CIT	1.21-1.00 (↓17%)	120 min	↑ 5.7-fold; 17 $pmol \cdot L^{-1}$	5-15 min	↑ 250%; 7 $pmol \cdot L^{-1}$
(1990)						
Gundberg *et al.*	iv CIT (pulse)	NS	30 min	NS	NS	NS
(1991)*	iv CIT (continuous)	NS	30 min	NS	NS	NS
Schwarz *et al.*	iv CIT	1.24-1.05 (↓15%)	120 min	↑ 5.2-fold; 17 $pmol \cdot L^{-1}$	5-10 min	↑ 2.5-fold; 7.5 $pmol \cdot L^{-1}$
(1992)	iv CC then CIT	1.23-1.50 (↑22%) CC	120 min CC / 120 min CIT	↓75%; 0.3 $pmol \cdot L^{-1}$	~15 min	↓75%; 0.3 $pmol \cdot L^{-1}$
		then 1.50-1.27 (↓15%) CIT		↑ 8.5-fold; 6 $pmol \cdot L^{-1}$	~20 min	↑ 3.1-fold; 1 $pmol \cdot L^{-1}$
Sørensen *et al.*	iv Ca	1.25-1.49 (↑ 20%)	120 min	↑ 2.7-fold; 2.5 $pmol \cdot L^{-1}$	30 min	↑ 2.7-fold; 2.5 $pmol \cdot L^{-1}$
(1992)						

Study	Method	iCa level ($mmol \cdot L^{-1}$)	Duration	Maximum PTH change (%change; level)	Time to max/min PTH	End PTH (% change, level)
Ramirez *et al.*	iv CG	NS	120 min	↓ 76%	50 min	NS
(1993)	iv CIT	↓0.21	120 min	↑396%; 33 $pg \cdot mL^{-1}$	30 min	NS
Schwarz, (1993)	iv CIT	↓2%	10 min	↑100%; NS	7 min	↑100%; NS
		↓5%	10 min	↑350%; NS	7 min	↑350%; NS
		↓7%	10 min	↑500%, NS	7 min	↑500%, NS
		↓11%	10 min	↑550%; NS	7 min	↑550%; NS
		↓16%	10 min	↑1100%, NS	7 min	↑1000%; NS
	iv CG	↑1.5	10 min	↓40%; NS	10 min	↓40%; NS
		↑4%	10 min	↓55%; NS	10 min	↓55%; NS
		↑8%	10 min	↓75%; NS	10 min	↓75%; NS
Schwarz *et al.* (1993)*	iv CIT then iv Ca	↓0.21 then ↑0.21		↑ 4-7 fold; ns; ↓ 75%, NS		↑ 2-3 fold, NS
Schwarz *et al.* (1994)	iv CIT then Ca	↓ 0.2 then ↑ 0.2	NS	↑ NS; 3.4-19.1 $pmol \cdot L^{-1}$	5-10 min	↑ NS; 8.6 $pmol \cdot L^{-1}$
				↓ NS; 0.9 $pmol \cdot L^{-1}$	NS	↓ NS; NS
Sørensen *et al.* (1994)*	iv Ca	↑ 0.25-0.30	120 min	↓92%; 0.6 $pmol \cdot L^{-1}$	NS	↓ NS; NS
Saha *et al.*	iv Ca	↑	120 min	↓ns, 0.8 $pmol \cdot L^{-1}$	NS	NS
(1997)*	iv CIT	↓	120 min	↑ 4.8-7.2-fold; 18.9 $pmol \cdot L^{-1}$	10 min	↑2-fold, NS

Study	Method	iCa level ($mmol \cdot L^{-1}$)	Duration	Maximum PTH change (%change; level)	Time to max/min PTH	End PTH (% change, level)
Schwarz *et al.* (1998)	iv CIT then iv CC then iv CIT	↓ 0.2, baseline, ↓ 0.2	75 min, 0, 10 or 30 min, 75 min (160-180 min)	↑ 6-16 fold; ↓ ~10%; ↑ 2-4 fold	5-10 min, 20 min, 20 min	↑2-4 fold; 0.8 $pmol \cdot L^{-1}$; ↑2-4 fold
	iv CIT	↓ 0.2 then ↓ 0.4	90 min, 40-55 min (130-145 min)	↑ 4.7-9 fold; ↑ 2.3-4.5	5-10 min, 10-20 min	↑2-4 fold; ↑2-4 fold
	iv CIT then iv CC then iv CIT then iv CC	↓ 0.2 then ↑ 0.2 then ↓ 0.2 then ↑ 0.2	75 min, 30 min, 60 min, 45-75 min (210-240 min)	↑ 6-17 fold; ↓~10%; ↑0.5-5 fold; ↓~10%	5-10 min; 20-45 min; 30-40 min; ~20 min	↑ fold; ↓~10%; 2-5 fold; ↓~10%

* abstract only; ↑, increase; ↓ decrease; ↔, unchanged; Ca, calcium; CC, calcium chloride; CG, calcium gluconate; CIT, citrate; EDTA, ethylenediaminetetraacetic acid; iCa, ionised calcium; iv, intravenous; NS, not stated; PTH, parathyroid hormone.

The studies described above do not discriminate between the effects of a change in the iCa concentration and the absolute concentration – be it higher or lower – itself. To examine this, Schwarz *et al.* (1992) induced hypercalcaemia and PTH suppression and followed it immediately by a citrate clamp. During the citrate clamp, iCa concentrations rapidly decreased from 1.50-1.27 $mmol \cdot L^{-1}$ and, in doing so, returned close to baseline levels. This decrease was accompanied by a 5 to 9-fold increase in PTH taking PTH levels to twice that seen prior to the induction of hypercalcaemia. This demonstrated that it is the decrease in blood ionized calcium, not the absolute concentration of ionized calcium itself that is associated with the PTH response. Schwarz *et al.* (1992) also concluded that the PTH response to hypocalcaemia has two distinct phases; the first, a release of preformed PTH from existing cellular depots and the second, an absolute iCa concentration-dependent secretion of newly synthesised PTH.

It is also possible that the rate of change in iCa determines the PTH response. In one early study, there was no difference in the peak PTH response to the same level of absolute hypocalcaemia induced over 60 or 120 min (Brent *et al.*, 1988). Subsequently, higher PTH peaks are reported with short, rapid, stepwise decreases in iCa interspersed with short but stable periods of hypocalcaemia compared with a generally linear decrease in serum iCa over the same 120 min period (Grant *et al.*, 1990). Despite this, however, the PTH level at the end of both inductions was similar and, as serum iCa tended to lower during the rapid decrease protocol, it is difficult to determine whether the higher PTH peaks result from a rate-of-change effect or from the greater degree of hypocalcaemia. Subsequently, decreases in iCa ranging from 2 to 16% over the same 10 min period are associated with dose-dependent decreases in PTH ranging from 2 to 11-fold (Schwarz, 1993). Similarly, the suppression of PTH by hypercalcaemia is also dose dependent (Schwarz, 1993). Importantly, whereas most other studies have induced changes in iCa in excess of 0.1 $mmol \cdot L^{-1}$, this study shows that PTH release is sensitive to far more subtle changes in calcium. It also shows that the changes in iCa can induce changes in PTH after only 2 min.

Studies of the calcium – PTH relationship have shown the presence of hysteresis with respect to PTH secretion (Conlin *et al.*, 1989) with a higher PTH value for the same serum calcium concentration during the induction of hypocalcemia (1.06 +/- 0.02 $mmol \cdot L^{-1}$) than during the recovery from hypocalcemia. In contrast, PTH values are less during the induction of hypercalcaemia than during the recovery from hypercalcemia (Conlin *et al.*, 1989). Whilst the aggressive induction of hypocalcaemia results in a rapid increase in PTH, a subsequent lowering of iCa gives rise to no or a markedly blunted response suggesting that the hysteresis seen in the PTH-iCa relationship might be due to a depletion of PTH stores (Schwarz *et al.*, 1998). This explanation, however, would not explain why the effect is seen during episodes of hypercalcaemia. Additionally, Aguilera-Tejero *et al.* (1996) report similar peak PTH concentrations during repeated hypocalcemia episodes, suggesting that this

effect is not due to PTH depletion. Instead, this effect has been interpreted to be a protective mechanism to prevent an overcorrection in serum calcium (Felsenfeld *et al.*, 2007).

2.4.1 Other factors that affect the PTH response to hypocalcaemia

2.4.1.1 Vitamin D status

Two years on a vitamin D and calcium-deficient diet reduces 1,25(OH)D and iCa and increases basal PTH and the maximal PTH response to an episode of hypocalcaemia by 600% (Cloutier *et al.*, 1992). One month of treatment with calcitonin increased both 1,25(OH)D and iCa but had little effect on maximum PTH despite the complete restoration of basal PTH levels (Cloutier *et al.*, 1994). Even after a further 18 months of vitamin D treatment, maximal PTH remained 3-fold above baseline suggesting the secondary hyperparathyroidism (reflected by the high maximal PTH) remains unresolved a long time after the onset of treatment.

2.4.1.2 Increasing age

The age-related increase in basal PTH is well-described and may be attributable to a number of factors (Felsenfeld *et al.*, 2007). Compared with young women, maximal PTH values in response to hypocalcaemia were increased in elderly women and remained so despite decreased values for both measures in both groups following calcitonin (Ledger *et al.*, 1994). In men, however, no significant effect of age is reported on maximal PTH (Portale *et al.*, 1997; Haden *et al.*, 2000).

2.4.1.3 Phosphate levels

Both *in vitro* and *in vivo* evidence suggests PO_4 is a key regulator of the circulating calcium concentration and PTH-mediated calcium mobilization from bone. *In vitro*, an increase in phosphorus concentration in the medium reduced calcium efflux from bone and the calcemic action of PTH. Importantly, a strong effect was observed between a 1 mM and 2 mM phosphorus concentration, a range observed in humans and many animal species (Raisz and Niemann, 1969; Ramp and McNeil, 1978). Studies in rats consuming different amounts of dietary phosphorous show that PO_4 loading affects the dynamic relationship between calcium and PTH (Reviewed in detail by Felsenfeld and Rodriguez, 1999).

These studies have shown that both the equilibrium operating point (or set point) of the calcium-PTH system (Figure 2.2) and the ability of PTH to maintain a normal serum calcium are affected in renal failure. Additionally, when calcium is a function of PTH during a PTH infusion, phosphorus loading impaired the calcemic action of PTH in both healthy rats and those with renal insufficiency. Thus, in PO_4 loaded animals, more PTH is needed to maintain a normal serum calcium and, when PTH is infused, the serum calcium was less for similarly high PTH levels. The role of PO_4 in mediating PTH activity is supported by PTH infusion studies in humans that show a rapid decrease in serum

phosphorus and increase in urinary phosphorus before any change in serum calcium (Roelen *et al.*, 1989). There is also evidence of a role of PO_4 in the development of secondary hyperparathyroidism in both vitamin D deficiency and aging (Felsenfeld and Rodriguez, 1999).

2.4.2 The role of PTH in changes in bone turnover and bone mass

As described above, one mechanism by which PTH serves to protect serum calcium concentrations is by mobilising calcium from bone via the activity of osteoclasts. Consistent with this mode of action, the chronic elevation of systemic PTH levels are associated with negative alterations to bone turnover and bone mass. Conditions associated with hyperparathyroidism, be it primary or secondary in nature, result in increases in bone turnover as evidenced by increases in hydroxyproline and pyridinolines (Duda *et al.*, 1988; Uebelhart *et al.*, 1991; Seibel *et al.*, 1992), 1CTP (Brahm *et al.*, 1994) and plasma β-CTX (Okuno *et al.*, 2005; Oliveri *et al.*, 2005). These conditions are associated with bone loss (Chan *et al.*, 1992; Ooms *et al.*, 1995; Khan *et al.*, 2009) and increased risk of fracture (Alem *et al.*, 2000; Sakuma *et al.*, 2006; Vignali *et al.*, 2009).

The role of excess PTH in these conditions is supported by studies of artificially-induced increases in circulating PTH concentrations using infusions. A 24 h infusion of PTH increases 1CTP (Brahm *et al.*, 1994) and decreases P1CP (Brahm *et al.*, 1994) while P1CP is also decreased by a 16 h infusion, returning to baseline at 14 h post-infusion (Simon *et al.*, 1988). More recent studies have shown similar effects on serum NTX (Leder *et al.*, 2001 Lee *et al.*, 2006) and plasma β-CTX (Lee *et al.*, 2006) OC (Leder *et al.*, 2001) and P1NP (Lee *et al.*, 2006). Consistent with these changes in bone turnover markers, continuous infusions of PTH have negative effects on bone structural parameters including reduced trabecular connectivity density in mice (Iida-Klein *et al.*, 2005) and reductions in cortical porosity (Zhou *et al.*, 2001) and BMD in rats (Tam *et al.*, 1982).

2.4.2.1 Anabolic effects of PTH on bone

Despite its recognised effect on increasing bone resorption and bone loss, the anabolic effects of PTH have also been known for some time (Bauer *et al.*, 1929). It was not until 1980, however, that this effect was reported in humans (Reeve *et al.*, 1980) before Neer *et al.* (2001) showed that exposure to PTH also decreased the risk of vertebral and nonvertebral fractures. Previous studies had shown that the effect of PTH on bone was dependent on its mode of administration. In rats, as described above, a continuous infusion of PTH-(1-84) results in an increased apposition rate, and increases in both formation and resorption surfaces resulting in a net decrease in the trabecular bone volume (Tam *et al.*, 1982). In contrast, daily injections of PTH caused an increase in the bone apposition rate, accompanied by an increase in the formation surface but no increase in the resorption surface, resulting in a net increase in trabecular bone volume. Daily injections in dogs increased measures of bone formation – surface osteoid, plasma alkaline phosphatase activity, and skeletal accretion rate of calcium – and resorption – number of osteoclasts, resorption surfaces – as well as iliac trabecular bone

volume. In contrast, continuous infusions increased osteoclast surfaces but had no effect on skeletal accretion rate of calcium or iliac bone volume (Podbesek *et al.*, 1983). In a series of investigations in rats, Hock and Gera, (1992) showed that intermittent and continuous PTH both increased bone formation independently of effects on bone resorption, but only intermittent PTH was able to consistently increase bone mass.

In humans, short-term intermittent PTH via subcutaneous injection has been shown to prevent spinal bone loss in women rendered estrogen deficient by a long-acting gonadotropin-releasing hormone (GnRH) (Finkelstein *et al.*, 1994). Subsequently, longer-term intermittent use prevents bone loss from the proximal femur and total body, and increases lumbar spine BMD in young women with GnRH analog-induced estrogen deficiency and increases bone mass in men (Orwoll *et al.*, 2003). Daily administration of PTH peptide 1-34 reduces vertebral and non-vertebral fracture risk in postmenopausal women with osteoporosis (Neer *et al.*, 2001) while the magnitude of vertebral fracture reduction after 18 months of PTH therapy is similar to that observed after one year of antiresorptive therapy (Harris *et al.*, 1999).

These positive outcomes for BMD are reflected in changes in bone turnover markers. Markers of bone formation (P1CP and bone ALP) are increased after one month of PTH therapy followed by increases in bone resorption markers at 3 months (DPD/Cr) and 12 months (NTX/Cr) with changes in bone formation markers at one month correlating with changes in bone structure after 22 months of therapy (Dobnig *et al.*, 2005). Chen *et al.* (2005) showed a similar pattern of change in bone formation and resorption markers and that increases in P1CP at 1 month and P1NP at 3 months were the most sensitive and accurate predictors of the lumbar spine BMD response to PTH therapy. The increase in bone formation prior to the increase in bone resorption has been termed the 'anabolic window' a period of time when the actions of PTH are maximally anabolic (Rubin and Bilezikian, 2003). More recently, frequent measures of bone turnover markers in the early stage of treatment has shown that this 'window' is larger still, with an 8% increase in P1NP after only 2 days with OC, P1NP, P1CP and OC increased 20% by 9 days, with plasma β-CTX and NTX suppressed for the first 19 days of therapy (Glover *et al.*, 2009).

Precisely how intermittent PTH achieves an increase in bone formation, bone structure and quality remains to be completely elucidated but it may relate to the change in serum PTH with the different regimes. Intermittent PTH treatment results in much higher serum PTH levels at peak than does continuous treatment (15,000 *vs* 600 $pg \cdot mL^{-1}$) (Dobnig and Turner, 1997). However, despite the large increase with a single injection, as PTH clearance is rapid, serum PTH quickly returns to basal levels. Compared to a prolonged elevation in PTH, this different pattern of change may have different effects on key regulators in the PTH signalling pathway (*e.g.* cAMP) leading to different expression patterns of PTH-regulated genes (Qin *et al.*, 2004). Indeed, Frolik *et al.* (2003) showed that pharmacokinetic

profiles indicate that the anabolic or catabolic response of bone to PTH is determined most strongly by the length of time each day that serum concentrations of PTH remain above basal levels.

With intermittent PTH therapy, bone formation markers are increased before those of resorption suggesting an uncoupling of bone formation from resorption. However, it is becoming increasingly apparent that the coupling may, in fact, remain intact (Martin and Sims, 2005). Holtrop *et al.* (1979) showed that an injection of PTH in young rats transiently activated osteoclasts *in vivo* within 30 min and, at high doses, was followed by an increase in the number of osteoclasts. A single injection of PTH results in a transient increase in mRNA for RANKL and a decrease in that for OPG. The maximum effect is seen at 1 h returning to control levels within 2 h, and it has been suggested that a subtle or transient increase in osteoclast formation or activation might be needed to prepare the bone surface for new bone formation (Ma *et al.*, 2001; Oniya *et al.*, 1995). More recently, a single subcutaneous injection of PTH increases plasma β-CTX after 60 min with levels increased 80% from baseline at 3 h, supporting the concept that osteoclast-mediated resorption is involved in the anabolic effects of PTH (Zikán and Stepan, 2008). The importance of bone resorption in the anabolic effects of PTH are supported by studies of combination therapy that show a blunted anabolic effect when PTH therapy is preceded by (Ma *et al.*, 2003) or given simultaneously with (Black *et al.*, 2003; Finkelstein *et al.*, 2003) antiresorptives. It is likely that the anabolic effects of PTH impacts osteoblasts, bone-lining cells, osteocytes, and osteoclasts, and activates multiple pathways that collectively result in the stimulation of bone formation (Figure 2.6) (Khosla *et al.*, 2008).

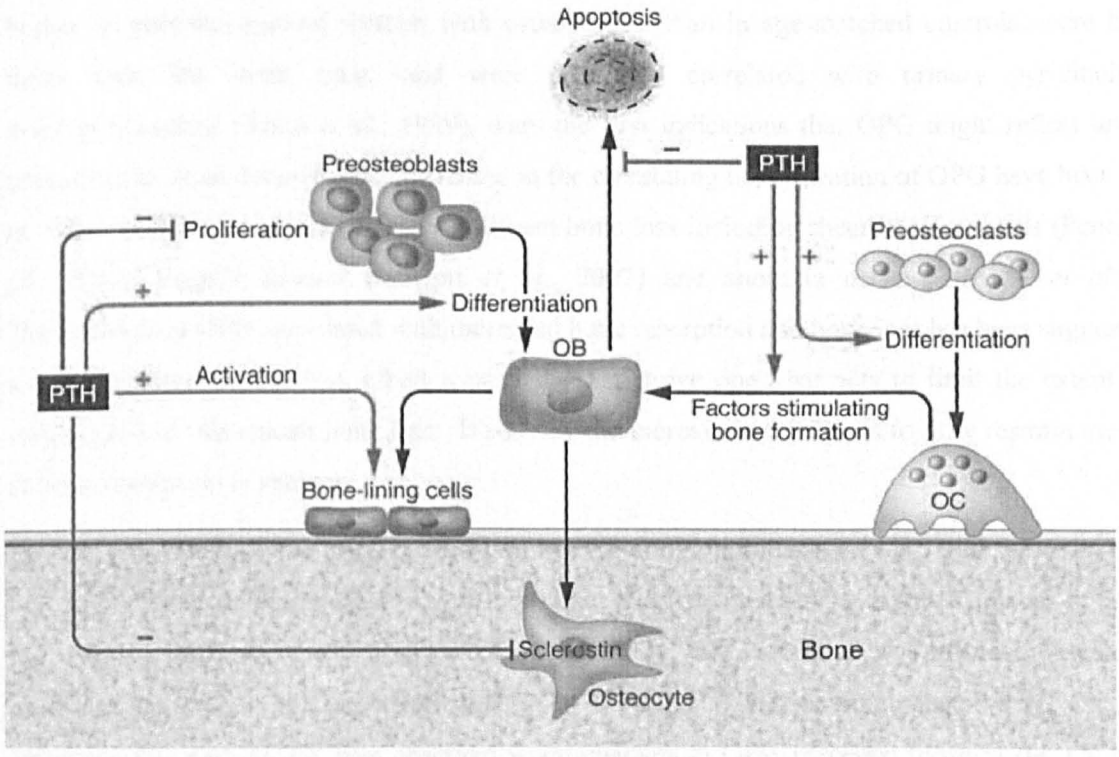

Figure 2.6 Potential cellular targets for the anabolic effects of PTH. *From Khosla et al. (2008).*

2.5 RANKL, RANK and OPG in the molecular control of bone turnover

An important development in the understanding of the molecular control of bone turnover has come with the identification of a pathway involving a family of tumour necrosis factor (TNF) receptor (TNFR)/TNF-like proteins: osteoprotegerin (OPG), receptor activator of NF-κB (RANK), and RANK ligand (RANKL) (Simonet *et al.*, 1997; Tsuda *et al.*, 1997; Lacey *et al.*, 1998; Yasuda *et al.*, 1998a, 1998b). In bone, RANKL-RANK interaction, stimulated by macrophage colony stimulating factor (M-CSF), has been shown to be the essential signals required to induce osteoclastogenesis, while the binding of soluble OPG to RANKL prevents RANKL-RANK interaction, and inhibits the formation of osteoclasts (Tsuda *et al.*, 1997). The levels of expression of RANKL and OPG may be modulated by numerous factors to control the rate of formation of osteoclasts and, importantly, nearly all osteotropic hormones and local factors produced in the bone micro-environment that have been shown to influence osteoclast activity, mediate their actions indirectly via RANKL/OPG expression (Hofbauer and Heufelder, 2001), further demonstrating their fundamental role in the control of bone turnover. Subsequently, RANKL, RANK and OPG have been found to be involved in the aetiology of various pathological conditions affecting bone including rheumatoid arthritis, Paget's disease, postmenopausal osteoporosis as well as malignant bone diseases (Buckley and Fraser, 2002).

2.5.1 Circulating OPG as a marker of bone metabolic activity

Soon after the identification of the RANKL-RANK-OPG axis, OPG was detected in human serum (Hsieh *et al.*, 1998). The subsequent findings that circulating OPG concentrations were significantly higher in post-menopausal women with osteoporosis than in age-matched controls, were higher in those with low bone mass, and were positively correlated with urinary pyridinoline and deoxypyridinoline (Yano *et al.*, 1999), were the first indications that OPG might reflect underlying alterations in bone remodelling. Increases in the circulating concentration of OPG have been reported in other conditions associated with significant bone loss including rheumatoid arthritis (Feuerherm *et al.*, 2001), Paget's disease (Martini *et al.*, 2007) and anorexia nervosa (Misra *et al.*, 2003). The increase in OPG associated with increased bone resorption and bone loss has been suggested to be a compensatory mechanism, albeit a partially ineffective one, that acts to limit the extent of bone resorption and subsequent bone loss. If so, why the increase in OPG fails to fully restrain the increase in bone resorption is unclear.

Elsewhere, cross-sectional studies report an inverse correlation between OPG and serum PTH in both men over 40 (Szulc *et al.*, 2001) and in both men and women aged 19 to 96 (Kudlacek *et al.*, 2003). In a recent carefully controlled circadian rhythm study, OPG secretion was inversely related to both PTH and plasma β-CTX secretion over a 24-h period. The maximal negative cross-correlation between OPG and β-CTX was observed when changes in OPG preceded those in β-CTX by 3 h in premenopausal women, and 2 h in postmenopausal women (Joseph *et al.*, 2007).

Pharmacological treatments that target bone cells also modulate circulating OPG. Human PTH (1–34) administered to postmenopausal women with glucocorticoid-induced osteoporosis decreases the circulating OPG concentrations (Buxton *et al.*, 2004). OPG concentrations are also decreased in Paget's patients treated with the bisphosphonate pamidronate (Martini *et al.*, 2007) as well as in previously untreated post-menopausal osteoporosis treated with alendronate and risedronate, where changes in OPG correlated with changes in the BMD response (Dobnig *et al.*, 2006).

Although early studies indicating a negative association with bone mass and a positive one with bone turnover markers (Yano *et al.*, 1999) suggested that circulating OPG may have the potential to be a sensitive biochemical indicator of bone cell and disease activity, subsequent studies have reported inconsistent findings regarding these relationships. These relationships include:

- positive correlations with serum markers of resorption and formation, and with urinary markers of resorption (Yano *et al.*, 1999);
- positive correlations with urinary markers of bone resorption but no correlations with serum bone formation markers or BMD (Szulc *et al.*, 2001);
- positive correlations with serum markers of bone formation with a borderline positive correlation with BMD (Indridason *et al.*, 2005);
- negative correlations with serum markers, no correlations with urinary markers and positively related to BMD (Rogers *et al.*, 2002);
- a negative correlation relation with a serum marker of bone formation but unrelated to BMD (Browner *et al.*, 2001) and;
- no correlation with serum markers, urinary markers or BMD (Khosla *et al.*, 2002).

These discrepancies might be explained by the cross-sectional design of these studies that do not capture dynamic changes in OPG that occur with the onset and progression of alterations to bone metabolism.

Findings from an increasing number of studies support the interpretation that the increase in OPG is a compensatory response to increased bone resorption (Yano *et al.*, 1999). Basal OPG levels do not consistently correspond well with measures of bone quality and bone turnover marker levels. For example, Han *et al.* (2005) report that, despite bone turnover markers correlating with BMD and with each other, OPG was not associated with either BMD or bone turnover in osteoporotic women or those with high bone turnover. Kim *et al.* (2008b) report a similar lack of associations. Other studies report no relationship between OPG and bone density in a healthy population aged 19 to 96 y (Kudlacek *et al.*, 2003) or in perimenopausal (Holecki *et al.*, 2007) or elderly women (Browner *et al.*, 2001), while in a study of acromegalic patients, bone resorption markers were markedly elevated but OPG levels were normal (Ueland *et al.*, 2001). In obese women, serum OPG was lower but BMD

higher compared to healthy controls (Holecki *et al.*, 2007; Holecki *et al.*, 2008). OPG has even been positively associated with BMD (Rogers *et al.*, 2002).

If OPG is indeed positively related to the rate of bone resorption, then changes in its levels might be expected to accompany changes in markers of bone resorption (and associated changes in BMD). However, again, there is a lack of correspondence between changes in OPG and changes in BTM and BMD in their short and long term response to both pharmacological and physiological interventions. Six months of treatment with bisphosphonates or teriparatide decreased and increased BTM respectively but neither therapy affected OPG levels (Anastasilakis *et al.*, 2008a). Likewise, 12 months of teriparatide (Anastasilakis *et al.*, 2008b) or 12 weeks of bisphosphonates (Choi *et al.*, 2008) have no effect on OPG despite significant increases and decreases in bone turnover respectively. In two studies, 6 months of bisphosphonate therapy decreased BTM levels and increased BMD but *increased* rather than decreased OPG (Dobnig *et al.*, 2006; Dundar *et al.*, 2009).

In the study of Dobnig *et al.* (2006), OPG levels after 12 months of BP treatment were positively better correlated to BMD changes than those of plasma β-CTX. Similarly, Hampson *et al.* (2003) report significantly better BMD responses in bisphosphonate treated patients with positive changes in OPG levels while patients with reductions in OPG had a less favourable outcome. Although the interpretation of the relationship between OPG and bone resorption following treatment with antiresorptives and teriparatide may be complicated by teriparatide acting on osteoblasts and antiresorptives acting on both osteoclasts and osteoblasts, similar findings have been reported following estrogen plus progestogen therapy (Kim *et al.*, 2008b). In another non-placebo controlled study, although a decrease in OPG with hormone therapy was observed, and this fall correlated with the fall in plasma β-CTX, it was not associated with the change in BMD (Han *et al.*, 2005). Choi *et al.* (2008) report no significant change in OPG despite a 37% fall in plasma β-CTX levels with 12 weeks of once-weekly alendronate.

Responses to physiological interventions are equally contradictory. Abrupt weight loss is known to increase bone resorption as evidenced by an increase in plasma β-CTX levels but OPG levels are decreased rather than increased (Holecki *et al.*, 2008). Additionally, the 50% reduction in plasma β-CTX levels following an oral glucose tolerance test (OGTT) is accompanied by only a 10% decrease in serum OPG while in diabetes patients there is no decline in OPG with OGTT despite a 40% decrease in β-CTX (Chailurkit *et al.*, 2008). As local expression of OPG mRNA levels in iliac bone biopsies has been correlated with increased fracture susceptibility (Abdallah *et al.*, 2005), taken together, these studies question the strength of the relationship between OPG and bone turnover.

2.5.2 The possible contribution of non-bone sources to circulating OPG concentrations

Much of this variation in relationships between OPG might also, in part, be due to the broad expression of OPG in human tissue as, in addition to bone, OPG is expressed in the kidney, lung and heart (Simonet *et al.*, 1997) and is regulated by a variety of hormones and cytokines. As such, it remains to be clearly established to what extent circulating OPG is an accurate reflection of local OPG production in bone (Simonet *et al.*, 1997; Browner *et al.*, 2001). There is gathering evidence that OPG, in conjunction with RANKL and its receptor RANK, are a common link between bone and the immune and cardiovascular systems (Doherty *et al.*, 2004). This link would provide a basis for the idea that chronic diseases such arthrosclerosis (Libby, 2002) and diabetes (Dandona *et al.*, 2004) are driven be inflammatory mechanisms. Thus, the age-related increase in OPG may be an adaptive response to onset of a low-grade systemic inflammation (Ballou *et al.*, 1996; Bruunsgaard *et al.*, 1999; Dobbs *et al.*, 1999). Consistent with this, OPG levels are elevated in inflammatory bowel conditions (Franchimont *et al.*, 2004; Moschen *et al.*, 2005) and familial Mediterranean fever (Yuksel *et al.*, 2009).

Inflammation also appears to underlie the pathology of insulin resistance and diabetes (Dandona *et al.*, 2004). OPG is found in the walls of the vasculature in concentrations similar to that in bone (Olesen *et al.*, 2005) and concentrations are elevated in patients in diabetes (Browner *et al.*, 2001; Knudsen *et al.*, 2003) and correlate with cardiovascular mortality (Browner *et al.*, 2001) and coronary artery narrowing (Schoppet *et al.*, 2003). Although diabetes is associated with hyperglycemia suggesting the increase in OPG might be related to increased blood glucose, Knudsen *et al.* (2007) observed no increase in OPG during a hyperglycaemic (15 $mmol \cdot L^{-1}$) clamp and OPG concentrations actually decreased suggesting no role of hyperglycemia *per se*. In this study of Knudsen *et al.* (2007), the decrease in OPG did correlated with the increase in insulin, a finding corroborated later by Jørgensen *et al.* (2009) who report an ~20% decrease in OPG at the end of 240 min of euglycemic-hyperinsulinemia (~400 $p \cdot mol^{-1}$). These studies suggest that processes other than the metabolic activity in bone contribute, at least in part, to OPG concentrations.

2.5.3. The measurement of RANKL in the interpretation of changes in OPG

As the balance between RANKL and OPG production in the bone microenvironment regulates RANK-RANKL binding and thus osteoclastogenesis, it is likely that the OPG to RANKL ratio, rather than the absolute activity of either factor, is the key determinant of the extent of bone resorption activity and the subsequent change in bone quality. Thus, a change in this ratio might result from fluctuations in RANKL alone, OPG alone, or differences in the magnitude (or direction) of changes in both. If OPG is indeed a reflection of local OPG activity, any interpretation of changes in its concentration is incomplete without a measure of circulating RANKL and the calculation of the OPG to RANKL ratio.

Most RANKL activity is provided by its expression on the osteoblast cell surface. As current assays for circulating RANKL measure the soluble form of RANKL, they lack sufficient sensitivity as soluble RANKL is unstable, is degraded rapidly, or binds to OPG to form large stable conglomerates (Chen *et al.*, 2003). Although many studies have attempted to measure RANKL and report the OPG to RANKL ratio, RANKL assays have been unable to measure any RANKL in between 41% and 90% of human subjects (Hegedus *et al.*, 2002; Alvarez *et al.*, 2003; Naylor *et al.*, 2003b; Szalay *et al.*, 2003; Mezquita-Raya *et al.*, 2005; Chaparro, 2008) including 55% of osteoporotic women (Mezquita-Raya *et al.*, 2005) who would have elevated bone turnover. Other authors appear to have had similar problems with the RANKL assay (Joseph *et al.*, 2007). If RANKL is undetectable in a high percentage of samples, providing the OPG to RANKL ratio could be misleading and result in inappropriate conclusions being drawn.

2.6 The effect of exercise on bone metabolism

2.6.1 The structural optimisation of bone in relation to its biomechanical function

In order to be an effective scaffold, the bones of the skeleton must serve as appropriate levers for muscles to contract against. Muscles typically at negative mechanical advantage, an arrangement that provides an increased range of movement but also increases the muscular force (and thus the joint force) required to move the lever. To remain structurally effective, bones are structurally optimised to distribute these increased forces without compromising their mechanical efficiency. In long bones at least, this is achieved with broadened bone ends to reduce stresses, and trabecular bone beneath the joint surface transitioning into the long bone cortex, allowing bones to support loads while remaining relatively light.

A large section of long bones can be considered as a thick-walled tube and, under physiological loading conditions, this tube will bend (Rubin and Lanyon, 1982). The deflection of a beam under bending is given by:

$$ML^2/8EI$$

where M is the bending moment, L is the length of the beam, E is the Young's Modulus and I is the second moment of area. Resisting the bending moment of a beam can be increased by reducing L, stiffening the beam material and increasing I, with the latter option best applying to bone. In the case of a tubular bone, I is equal to:

$$\pi/4\ (r_o^4 - r_i^4)$$

where r_o is the outer radius and r_i is the inner radius. High I values and low bone mass produce structurally efficient bones and the ratio of diameter to cortical thickness is thus a measure of structural efficiency. Although a solid bone would produce a higher (less than 10%) I value, the

presence of a marrow cavity reduces mass by more than 15% resulting in improved structural efficiency.

Bone achieves structural optimisation by forming tissue in areas of high stress but not in areas of low stress where its presence offers no structural advantage (*i.e.* mass that does not contribute to resistance to deformation). For example, during axial compression of the rat ulna (a long bone), strains are higher on the medial side than the lateral side and the pattern of bone formation resembles the strain distribution, with a greater amount of bone formed on the medial surface (Robling *et al.*, 2002). Despite only a 5% increase in BMD, loaded bones showed a 69% increase in second moment of area, a 64% increase in strength and a 94% increase in energy absorbed before fracture, demonstrating the importance of biomechanical alterations in improvements in structural efficiency. In trabecular bones, particularly vertebral bodies, the proximal tibia and the calcaneus, trabeculae tend to align with maximum stresses resulting in an in stiffness and strength in the direction of alignment. As in long bones, this is achieved without increasing mass and thus results in an improvement in structural efficiency.

2.6.1.1 Functional adaptation of bone structure – studies in animals

Much of the information regarding the effects of mechanical loading on bone has come from *in vivo* animal studies that have utilised axial compression of long bones and these experiments have revealed important characteristics of the mechanical induction of osteogenesis. The advantage of this experimental model is that it allows the effects of 'pure' mechanical loading to be investigated. This is not possible in humans as there are no forms of physical activity that are characterised by the repetition of a single, unidirectional mechanical load. For example, in all forms of endurance exercise, multiple mechanical loads are imposed on bone either through a combination of ground reaction forces (GRF) and muscular forces (*e.g.* running) or through forces produced by multiple muscles (*e.g.* cycling, rowing, resistance exercise). Additionally, the magnitude and direction of these forces will also continuously vary during the course of a single bout of exercise.

In these types of exercise – and indeed with resistance exercise – it is also impossible to separate the effects of pure mechanical loading from changes in metabolic factors such as hormones (*e.g.* PTH, IGF-1), which might influence bone in an endocrine manner. Even with an exercise as simple as a drop jump, in addition to the mechanical load imposed by gravity, bone likely also experienced muscular forces that act to control the motion of the lower limb during landing. Depending on the height of the jumps and the number performed in a single session, such exercise might also have a significant metabolic component. Importantly, irrespective of the type of exercise, when studying the effects of repeated bouts exercise over periods ranging from weeks to years, all studies in humans must consider the effects of factors such as diet, sunlight exposure and unprescribed exercise, all of which might influence bone in the longer-term. In contrast, *In vivo* loading

experiments in animals are able to provide much tighter control of these potentially confounding factors.

Consistent with the importance of structural optimisation in the biomechanical function of bone, it is also capable of significant functional adaptation in response to changes in its biomechanical environment. This adaptation can be summarised as:

- loading increases bone formation on the periosteal bone surface and inhibits net bone loss on the endocortical and trabecular surfaces;
- unloading suppresses periosteal bone formation and accelerates bone loss on endocortical, trabecular and intracortical bone surfaces;
- bone microstructure tends to align with peak forces.

Using implanted metal wires, Hert and colleagues demonstrated that osteoblasts respond to dynamic rather than static loading (Hert *et al.*, 1969; Hert *et al.*, 1972) suggesting that stress or strain alone cannot stimulate bone formation. Subsequently, bone formation has been shown to be proportional to strain rate (Turner *et al.*, 1995; Mosley *et al.*, 1998), which would be consistent with static loading – which has a strain rate of zero – having no effect on bone formation. Although strain itself is not the primary stimulus for bone formation, subsequent studies indicated that strains during dynamic loading needed reach a specified magnitude before bone formation is initiated (Chow *et al.*, 1993; Rubin and Lanyon, 1985; Turner *et al.*, 1994; Hsieh *et al.*, 2001) and, once this threshold was surpassed, bone formation correlated positively with peak strain magnitude (Rubin and Lanyon, 1985; Turner *et al.*, 1994; Hsieh *et al.*, 2001). Additionally, the strain threshold for bone formation in long bones varies according to location, with bone formation initiated at a lower strain magnitude at the distal end, which corresponds with the location of greatest strain (Hseih *et al.*, 2001).

The finding that dynamic loading was required for bone formation prompted investigations of the role of loading frequency in this response. These studies showed that loading frequency must exceed 0.5 Hz to achieve osteogenic effects (Turner *et al.*, 1994), while further experiments showed that increasing the frequency from 1 Hz to 10 Hz, reduced the strain required to initiate bone formation from 1820 $\mu\epsilon$ to 650 $\mu\epsilon$ (Hsieh and Turner, 2001). Interesting, high frequency (30 Hz), low magnitude (5 $\mu\epsilon$) loading increases bone formation increases trabecular bone volume but has no effect on cortical bone (Rubin *et al.*, 2001).

Using an in vivo loading model of isolated rooster ulnas, Rubin and Lanyon, (1984) demonstrated that bone formation occurred with only 36 loading cycles per day but there was no additional bone formation with 360 daily cycles suggesting that the sensitivity of bone is rapidly diminished during repetitive loading. Subsequent experiments showed that this sensitivity is restored during periods of rest and also that the magnitude of new bone formation increases when rest periods are lengthened so that the bone formation response to 4 bouts of 90 cycles separated by 4 h is twice that seen with one

bout of 360 consecutive cycles (Robling *et al.*, 2002). The addition of rest periods between individual cycles appears to have a similar effect, with rest periods ranging from 10 to 14 sec enhancing the osteogenic response compared with continuous, uninterrupted loading (Srinivasan *et al.*, 2002).

Having "time off" from loading has also been shown to improve mechanical sensitivity during longer-term periods of loading (Saxon *et al.*, 2005). In this study, loading was applied to rat ulnas for either 5 weeks followed by 10 weeks of time off, for 5 weeks followed by 5 weeks of rest and a further 5 weeks of loading, or 15 weeks of continuous loading. Bone formation increased in all three groups during the first 5 weeks of loading, but only in the "time off" group did bone formation increase during the last 5 weeks of the experiment. This group showed greater improvements in work to failure compared with both the 5 weeks and 15 weeks loading groups. Importantly, this study also demonstrated that the reduced skeletal response to long-term mechanical loading was not due to aging but rather a waning effect to long-term mechanical loading. Finally, using 15 weeks of either progressively decreasing loads, increasing loads, or a constant load, Schriefer *et al.*, (2005) showed that bone formation rates were increased only in the early weeks of loading and the degree of adaptation was proportional to the initial peak load magnitude. This finding is consistent with a previous study (Kim *et al.*, 2003) that reports significantly greater trabecular bone formation occurring within the first week of loading compared with period from 2 to 4 weeks, further highlights the importance of the early period of a new loading regime.

Studies of disuse have been equally informative in understanding the role of long-term loading on bone. Experiments in which dogs had one forelimb placed in a spica cast, report marked bone loss (Uhthoff and Jaworski, 1978; Jaworski *et al.*, 1980). In young, growing animals, appositional growth was suppressed at the periosteal surface resulting in a reduced cross-sectional area and lower second moment of inertia. In older and mature dogs, however, immobilisation was associated with accelerated remodelling and bone resorption, with most bone loss occurring at the endosteal surface. An increase in cortical porosity was also evident in these animals. Likewise, rapid trabecular bone loss from long bones is a common feature of disuse (Jaworski and Uhthoff, 1986).

In both growing and mature animals, bone loss is greatest at the distal end of the immobilised limb (Uhthoff and Jaworski, 1978; Jaworski *et al.*, 1980). Taken together with findings from loading experiments, where the strongest osteogenic effects are seen at the distal end, this finding suggests that the distal end of long bones is most responsive to a marked change in the mechanical environment. This may be due to the distal end being closer to the ground and thus loaded more directly or that the distal end is subject to greater interstitial fluid pressures which would enhance appositional bone growth. As with the osteogenic effects of loading, the effects of disuse appear to be self-limiting, with bone resorption initially increased but eventually returning to normal (Li *et al.*, 1990). Turner, (1999) has postulated these findings to represent 'cellular accommodation', a mechanism by which bone cells react strongly to changes in their mechanical environment but weakly to steady-state signals,

with the response eventually ceasing as cells accommodate to the new environmental conditions (Turner, 1999).

Although high stresses and strain rates typically result in increased bone formation and reduced bone turnover, excessive forces and deformations can result in damage to bone. In response to this damage, bone remodelling units are activated and intracortical bone remodelling is accelerated. This acceleration in remodelling occurs in species in which bone is osteonal and has an existing baseline of intracortical remodelling (*e.g.* dogs) and those where intracortical turnover is characteristically absent (*e.g.* rodents) (Bentolila *et al.*, 1998; Mori and Burr, 1993). These studies utilise 'fatigue loading' protocols, where loading is continued to a single stopping point based on loss of bone stiffness, determined from increases in ulnar compliance, defined as the increase in absolute displacement range during cyclic loading. Importantly, in the study of Bentolila *et al.*, (1998), the target fatigue level was not achieved in two animals and intracortical resorption was evident only in association with bone fatigue.

In addition to a reduction in stiffness, *in vivo* fatigue loading protocols are also associated with the development of linear mircocracks (Bentolila *et al.*, 1998; Mori and Burr, 1993; Burr *et al.*, 1985). However, whole bones fatigue lose large amounts of stiffness before linear microcracks became apparent (Burr and Stafford, 1991) suggesting that other changes must be involved to account for early increase in compliance. Indeed, subsequent studies showed that bone fatigue results in focal patches of bone with increased permeability to staining (Schaffler *et al.*, 1996; Boyce *et al.*, 1998; Bentolila *et al.*, 1998), which comprise very fine matrix microcracking at the sublamellar level. Unlike microcracks, which show a strong association with regions of intracortical resorption and which decrease markedly in number in the days following fatigue loading, regions of diffuse damage are weakly associated with resorption activity and show little decrease in their number (Bentolila *et al.*, 1998). More recently, Herman *et al.*, (2010) have shown that fatigue loading must result in microcracks to induce a remodelling response, with the presence of diffuse damage alone associated with no activation of resorption.

In addition to intracortical remodelling, fatigue loading also induces periosteal woven bone formation (Bentolila *et al.*, 1998; Hseih and Silva, 2002; Tami *et al.*, 2003). The location of maximal woven bone formation correlates with the site of peak strain during loading (Kotha *et al.*, 2004) which in turn corresponds with sites of fatigue crack formations (Tami *et al.*, 2003). At 7 days after fatigue loading, the area of new periosteal woven bone formation is proportional to the level of imposed damage (Uthgenannt *et al.*, 2007) suggesting that woven bone formation is damage dependent. However, it is unclear from these studies if the woven bone response is due to bone damage (*i.e.* a response to injury) or dynamic strain (*i.e.* an adaptive response). Using a creep loading model, Lynch and Silva, (2008) showed that bone damage created without dynamic strain also triggers woven bone formation, suggesting that the osteogenic response is largely a response to bone damage. Consistent with bone

format in other contexts that requires angiogenesis, woven bone formation with fatigue loading is preceded by a significant increase in perisoteal vascularity (Matsuzaki *et al.*, 2007; Wohl *et al.*, 2009), the magnitude of which is proportional to the mechanical stimulus (level of fatigue displacement) (Matsuzaki *et al.*, 2007). In fact, the woven bone response to fatigue damage is comparable to the intramembranous portion of fracture repair, being characterized by increases in the expression of genes associated with angiogenesis [vascular endothelial growth factor-A (VEGF), platelet-endothelial cell adhesion molecule-1 (PECAM-1)], cell proliferation and osteoblastogenesis [bone morphogenetic protein (BMP-2), homeobox msh-like 2 (Msx2), RUNX2, osterix (OSX), bone sialoprotein (BSP), osteocalcin, OC) (Wohl *et al.*, 2009). In a more recent study, IL-6 mRNA was increased (220-fold) 4 h after fracture and IL-11 peaked (73-fold increase) at 24 h post-loading, suggesting that IL-6 and IL-11 play a central role in initiating signalling events for remodelling and healing (Kidd *et al.*, 2010). Additionally, upregulation of cyclooxygenase-2 (COX-2), VEGF, OPG, stromal cell-derived factor-1 (SDF-1), BMP-2 and SOST preceded peak expression of RANKL suggesting a role for these factors in mediating directed remodelling.

Although fatigue loading results in damage, remodelling, and a decrease in mechanical properties, with time the woven bone response leads to a rapid recovery of whole-bone stiffness and strength (Hsieh and Silva, 2002) and even an increase in fatigue life at 12 days post-loading (Silva and Touhey, 2007). Even when loading is sufficient to produce a non-displaced fracture, by 14 days the woven bone response results in complete recovery of strength and stiffness, restoring mechanical properties to normal levels (Martinez *et al.*, 2010).

In summary, animal studies of have demonstrated bone's potential for osteogenic adaptation in response mechanical loading as well as identifying loading characteristics that appear to optimise this response. In combination with studies of disuse, which have demonstrated the importance of habitual loading in the maintenance of normal bone mass, these studies suggest that bone cells react strongly to marked changes in their mechanical environment but, when the subsequent environment remains unchanged (*e.g.* with continued loading in the same manner or with further disuse), this response soon diminishes and eventually ceases as cells accommodate to the new conditions. Importantly, these studies have also shown that when loading is excessive it results in damage, increased bone resorption and focal porosity, and a loss of importance mechanical properties. However, without additional loading, over time bone mounts an adaptive response, similar in part to the repair of frank fracture and characterised by angiogenesis and woven bone formation, which restores and even improves important mechanical characteristics. Taken together, these findings suggest that the manner in which bone responds to mechanical loading may be dictated by the intensity of mechanical signals and also by the period of time that bone has to adapt (or recover) between these signals.

2.6.2 The effect of acute exercise on bone turnover markers

With the availability of BTM and the existing knowledge of the effects, both positive and negative, of mechanical loading and physical activity on bone tissue, came an interest in investigating the effects of acute exercise on their concentrations. Previously, investigators had been limited to the use of scanning technologies such as DEXA, where the effects of chronic physical activity would only be evident after a period of months if not years. Alternatively, the effect of a single bout (Cunningham *et al.*, 1985) or a short period (Ljunghall *et al.*, 1988) of exercise could only be studied by measuring changes in circulating calcium and calcium regulating hormones including PTH that provided only indirect information regarding bone metabolism. The availability of BTM, however, through an examination of changes in their circulating concentrations during, and in the hours and days afterwards, has allowed the more direct study of the effects of acute exercise.

The first studies that examined the effects of acute exercise on BTM appeared in the late 1980's and, over the course of the following 20 years the BTMs examined in subsequent studies have reflected their availability. Whilst early studies measured non-specific markers such as Hyp and urinary PYD, the most recent studies have chosen more specific markers including plasma β-CTX and P1NP (Guillemant *et al.*, 2004; Tosun *et al.*, 2006; Maïmoun *et al.*, 2006). Thus, there is some logic in discussing previous studies in a chronological manner. A summary of these studies is provided in Tables 2.5 and 2.6.

2.6.2.1 Studies published in the 1980s and 1990s

In what appears to be the first study of the effects of acute exercise on BTM, Nishiyama *et al.* (1988) measured OC in male volleyball players and their sedentary counterparts before and after a 30 min run at what is described as a constant workload of 43-52% of the subject's maximum. They report an ~60% increase in OC in the sedentary males immediately after exercise that was no longer present at 60 min post-exercise. The volleyball players also showed an increase in OC but it was less pronounced (~20%) and only present at 60 min post-exercise. Their results suggested that exercise could have an anabolic effect on bone tissue although, as they did not measure any bone resorption markers it is not possible from their study to examine the net effect of exercise on bone turnover. Their study is also the first indication of a possible effect of habitual physical activity level on changes in BTM with acute exercise.

Table 2.5. Studies published in the 1980s and 1990s that measured changes in bone turnover markers in response to acute exercise

Study	Subjects	Age (mean)	Status/Activity	Mode	Duration/Intensity	FAST[1]	CON[2]	PV[3]	Findings
Nishiyama *et al.* (1988)	m (19)	22	Athletic (A) and non-athletic students (NA)	RUN	30 min @ 43-52% effort	NS	N	N	A (OC ↑ (1 h)) NA (OC ↑ (0 h))
Malm *et al.* (1993)	m (8) f (15)	30 40	Amateur runners	RUN	Marathon (3 h 56 min) (4 h 12 min)	NS	N	N	m (Hyp ↔; bone ALP ↔; OC ↓ (0 h, 1 d, 3 d) f (Hyp ↔; bone ALP ↓ (0 h, 1 d, 3 d, 5 d); OC ↓ (0 h, 1 d, 3 d, 5 d)
Virtanen *et al.* (1993)	m (9)	25	Sports training background	RES	3x CMJ, 3x 1RM leg extensions, 50x 1RM leg extensions, 3x CMJ	NS	N	N	P1CP ↓ (1 h), ↑ (2 d and 4 d)
Salvesen *et al.* (1994)	m (7) f (8)	25 21	cross-country runners	RUN	4 min @ 25%, 50%, 75% VO_{2max} followed by 9 min incremental to exhaustion	NS	N	N	m (1CTP ↔; OC ↔; P1CP ↑ (0.5 h)) f (1CTP ↔; OC ↓ (0.5 h); P1CP ↔)
Brahm *et al.* (1996)	m (10) f (10)	39 38	Regular exercisers	RUN	28 km (15 to 30) 15 km (5 to 30)	Y	N	N	1CTP ↑ (2 d); bone ALP ↔; OC ↓ (1 d); P1CP ↔ 1CTP ↔; bone ALP ↔; OC ↔; P1CP ↓ (1 d)
Kristoffersson *et al.* (1995)	m (7)	22	Elite ice hockey players	CYC	Wingate test (+ 60 sec of unloaded pedalling)	Y	N	N	1CTP ↔ P1CP ↔ OC ↔
Thorsen *et al.* (1996)	f (12)	68	Postmenopausal No regular training	WALK	90 min at 50% HR reserve	N	Y	N	P1CP ↑ at 24 and 72 h (13%) 1CTP ↓ at 1 h and ↑ at 72 h (15.6%)

Study	Subjects	Age (mean)	Status/Activity	Mode	Duration/Intensity	FAST[1]	CON[2]	PV[3]	Findings
Ashizawa *et al.* (1997)	m (10)	24	No resistance training for 2 years	RES	3 sets of 7 exercises 1 set at 60% 1RM 2 sets at 80% 1RM	NS	N	N	fDPD ↓ (1 h)
Brahm *et al.* (1997a)	m (10) f (10)	30 28	VO_{2max} = 56 VO_{2max} = 47	RUN	10-min @ 30%, 10 min @ 47%, 10-min @ 76%, VO_{2max} to exhaustion	N	N	Y	OC ↔; OC content ↓ 14% (0 h and 1 d) P1CP concentration ↑ (during); content ↑ (1 d) Total ALP concentration ↑ (during); content ↑ (1 d) 1CTP concentration ↑ (during and 1 d); ↑ content (1 d).
Brahm *et al.* (1997b)	m (6) f (6)	25 28	Not regular exercisers	RES	10 min @ 38%, 15 min at 61% and 5 min at 100% W_{max}	NS	N	N	P1CP ↑ with ex. and was released from working muscle at end of ex. 1CTP ↓ during recovery and release from working muscle at end of ex. OC ↓ at end of ex. and ↑ thereafter. bone ALP ↔ uptake and release; ↑concentration release at post ex.
Brown *et al.* (1997)	m (2) f (2)	22	Untrained	RES	50 reps @ max effort, of eccentric knee extension	NS	N	N	Hyp ↑ (2 d) Hyl ↑ (2 d, 5 d and 9 d) PYD ↔ total ALP ↔
Rong *et al.* (1997)	m (8)	23	Healthy	CYC CYC RES	45 min, 50% VO_{2max} 15 min, 85% VO_{2max} 85% of 3RM	NS	Y	Y	1CTP ↔ vs CON; OC ↔ vs CON 1CTP ↔ vs CON; OC ↔ vs CON 1CTP ↔ vs CON; OC ↔ vs CON

Study	Subjects	Age (mean)	Status/Activity	Mode	Duration/Intensity	FAST[1]	CON[2]	PV[3]	Findings
Thorsen *et al.* (1997)	f (14)	25	Inactive	RUN	45-min outdoor jogging @ 50% VO_{2max}	N	N	Y	P1CP ↓ (1 h); ↑ (1 d) 1CTP ↑ (1 d and 3 d)
Welsh *et al.* (1997)	m (10)	26	NS	WALK	30-min @ 60% HR_{max}	NS	N	N	OC ↔ bone ALP ↔ fDPD ↑ (2 d) fPYD ↑ (2 d)
Ashizawa *et al.* (1998)	m (14)	24	No resistance training for 2 years	RES	3 sets of 7 exercises 1 set at 60% 1RM 2 sets at 80% 1RM				fDPD ↓ 3 d) P1CP ↓ (1 d) bone ALP ↓ (2 d and 3 d) OC ↔ TRACP ↓ (1 d)
Brown *et al.* (1999)	m (4) f (5)	26	untrained	RES	50 reps @ max effort of concentric (C) or eccentric (E) knee extension	NS	N	N	C (total ALP ↔; HYP ↔) E (total ALP ↔; HYP ↔)

Study	Subjects	Age (mean)	Status/Activity	Mode	Duration/Intensity	FAST[1]	CON[2]	PV[3]	Findings
Crespo *et al.* (1999)	m (11) f (7)	26 28	M (2.5 ± 0.4 h) F (2.6 ± 0.4 h) for marathon	RUN	Marathon	NS	N	Hct	TRACP ↓ (0 h and 1 d) total ALP ↑ (0 h and 1 d)
Langberg *et al.* (1999)	m (6) f (1)	32	7 (4 to 12) h training per week	RUN	12 km.h^{-1} for 180 min outdoor	NS	N	Y	P1CP ↓ (0 h to 2 h; ↑ (3 d) 1CTP ↔

[1] were subjects fasted overnight prior to exercise? (Y, yes; N, no; NS, not stated; 2 h, for 2 h pre-exercise); [2] was a control group included?; [3] were bone markers concentrations corrected for changes in plasma volume (PV)?; ↑, increase; ↓ decrease; ↔, unchanged; 1CTP, c-terminal cross-linking telopeptides of type 1 collagen generated by matrix metalloproteinases; ALP, alkaline phosphatase; bpm, beats per min (heart rate); CMJ, counter-movement jump; CON, control group; CYC, cycling; f, female; fDPD, free deoxpridinoline; fPYD free pyridinoline; HR, heart rate; HR_{max}, heart rate maximum; Hyp, hydroxyproline; Hyl, hydroxylysine; m, male; OC, osteocalcin; P1CP, c-terminal propeptides of type 1 procollagen; RES, resistance; RM, repetition maximum; RUN, running; TRACP, tartrate-resistant acid phosphatise; VO_{2max}, maximum oxygen uptake (ml·kg^{-1}·min^{-1}); W_{max}, workload max; WALK, walking.

No further papers were published until 1993, when Malm *et al.* investigated the effect of a marathon (41.195 km) on Hyp, OC and bone ALP concentrations in amateur male and female runners (Malm *et al.*, 1993). OC levels were decreased in both groups immediately after the marathon and remained so at 3 days and 5 days post-exercise in men and women respectively, while bone ALP was decreased up to 5 days post-exercise in women but showed no change in men. Urinary Hyp excretion (in $mmol \cdot min^{-1}$) tended to be higher in both men and women from 1 to 3 days after the race but the increase was not significant. These findings indicate that a single bout of strenuous, long-duration exercise might suppress, rather and enhance bone formation.

In the same year Virtanen *et al.* (1993) measured serum PICP and Hyp before and after an acute bout of exercise consisting of countermovement jumps, maximal isometric and concentric contractions. They report decreased PICP at 1 h post exercise but then a significant increase above baseline at 2 days and 4 days of recovery. In contrast, they observed no change in Hyp over the same period, pointing to a positive effect of exercise on collagen synthesis.

1994 saw the publication of the first study to measure serum markers of both collagen synthesis (PICP) and degradation (ICTP) in response to the same acute exercise protocol (Salvesen *et al.*, 1994). In this study, well-trained men and women performed an incremental exhaustive running test lasting 21 min. Blood samples drawn 30 min after exercise indicated no effect of exercise on ICTP with a 12% increase in PICP in men but not women. Salvesen *et al.* (1994) also measured OC, but found the reverse pattern to that of PICP with an increase in women but not men. A year later, using the same markers, Kristoffersson *et al.* (1995) observed no effect of short-term, supramaximal exercise (Wingate test) when measured at 5 min and 60 min post-exercise.

1996 saw the two further studies measuring changes in PICP and ICTP in response to the same acute exercise protocol (Brahm *et al.*, 1996; Thorsen *et al.*, 1996). In the study of Thorsen *et al.* (1996), 12 postmenopausal women walked for 90 min at 50% of heart rate reserve and PICP and ICTP were measured at 1 h, 24 h and 72 h following exercise. They report an ~13% increase in PICP at 24 and 72 h after exercise. ICTP on the other hand was decreased ~14% at 1 h post-exercise but, like PICP, increased by a similar amount at 72 h.

Brahm *et al.* (1996) measured the same markers, as well as bone ALP, before and 24 h and 48 h after a running race of 15-30 km (males) and 5-30 km (females). Like Salvesen *et al.* (1994), they observed different effects of the race on men and women but observed decreases rather than increases in with PICP in women and OC in men. They also observed an increase in ICTP at 48 h post-exercise in men only.

The first studies to measure the urinary pyridinolines as markers of bone resorption were published in 1997 (Ashizawa *et al.*, 1997; Welsh *et al.*, 1997; Brown *et al.*, 1997). Ashizawa *et al.* (1997) measured urinary free deoxypyridinoline (fDPD) each hour for 4 h following a 45 min bout of strenuous resistance exercise. They report a transient decrease in fDPD (in $pmol \cdot min^{-1}$) at 2 h post-exercise that was no longer evident in the next sample. They did not, however, measure any markers of bone formation in response to this protocol. In the study of Welsh *et al.* (1997) young, healthy males provided 24 h urine samples before and after 30 min of brisk treadmill walking at a heart rate of ~60% of age-predicted maximum heart rate. When corrected for creatinine concentrations and bone mineral content (BMC), both free pyridinolines (fPYD) (25%) and fDPD (29%) were significantly increased from pre-exercise on the second day post-exercise. A similar magnitude and pattern of change was seen when pyridinolines were corrected for urine volume and BMC (expressed as $nmol \cdot day^{-1} \cdot g^{-1}$). Welsh *et al.* (1997) also measured OC and bone ALP but found no significant change in either marker up to 32 h following exercise.

Brown *et al.* (1997) examined changes in Hyp, Hyl and fPYD (corrected for creatinine), as well as total ALP following a single bout of maximal intensity eccentric contractions. They observed a transient increase in Hyp at 2 days post-exercise and an increase in Hyl at 2 days, 5 days and 9 days post-exercise with no change in either PYD or total ALP.

Ashizawa and colleagues performed a subsequent study of resistance exercise measuring fDPD in 24 h samples, and serum markers of bone formation from overnight fasted samples at 0800 h, up to 3 days post-exercise (Ashizawa *et al.*, 1998). They observed decreased fDPD output (in $nmol \cdot day^{-1}$) at 1 and 3 days post-exercise although this decrease was only significant on the third day. Although, unlike in their previous study, they do not also report their fDPD values corrected to creatinine, they report no effect of the exercise protocol on creatinine output (Ashizawa *et al.*, 1998). TRACP was also significantly reduced at 1 day post exercise while P1CP and bone ALP were transiently decreased at 1 day and 2 days post-exercise respectively.

Both Thorsen *et al.* and Brahm *et al.* published further studies using P1CP and 1CTP in 1997 (Brahm *et al.*, 1997a; Brahm *et al.*, 1997b; Thorsen *et al.*, 1997). Thorsen *et al.* (1997) examined changes in P1CP and 1CTP in young healthy women following 45 min of jogging at 50% VO_{2max}. Similarly to their 1996 study, they report a significant increase in 1CTP and P1CP following exercise when measured at 24 h and 72 h, although P1CP was lower than its pre-exercise value at 1 h post-exercise. In contrast to the 1996 study, however, that found no effect of exercise on OC, they observed a transient increase in OC at 1 h post-exercise. Brahm *et al.* (1997a) examined changes in 1CTP and P1CP during and following a 35 min, incremental, exhaustive running protocol. They observed increased 1CTP at the end of exercise that had returned to baseline at 30 min post-exercise but was increased again at 24 h post-exercise. P1CP and total ALP were also increased late in exercise, but not thereafter, whereas OC was unchanged throughout exercise and recovery. In their second 1997 study,

Brahm *et al.* (1997b) used a similar protocol but with subjects completing one-legged knee extensor exercise rather than running. 1CTP was unchanged during exercise but was significantly decreased at 60 min post-exercise, whereas P1CP was increased throughout exercise but not thereafter. Bone ALP was increased at the end of exercise only while OC was transiently decreased immediately after exercise only. In the same year, Rong *et al.* (1997) compared changes in 1CTP and OC to three different exercise protocols: 45 min cycling at 55% VO_{2max}, 15 min cycling at 85% VO_{2max} and leg resistance exercise at 85% of three-repetition max (RM). Compared to a resting control group, they report no difference in post-exercise concentrations of either marker with any of the exercise protocols although, when compared to pre-exercise values, only the exercise groups showed a significant decline in 1CTP and OC at 4 h post-exercise.

In 1999, Crespo *et al.* measured total ALP and TRACP in elite male and female marathon runners completing a competitive marathon (Crespo *et al.*, 1999). They observed a significant increase in total ALP immediately and 24 h after the race and decreased TRACP at both time points. Also in 1999, Langberg *et al.* examined changes in P1CP and 1CTP associated with a 36 km treadmill run. In five samples drawn in the first 2 h after the run, P1CP was significantly decreased but was increased from pre-exercise values in five further samples taken at 72 h post-exercise (Langberg *et al.*, 1999). In contrast, no significant changes in 1CTP were observed. In a follow-up to their 1997 study, Brown *et al.* (1999) compared an acute bout of maximal eccentric exercise – identical to that used in the 1997 study – to an equivalent bout of eccentric exercise. Unlike the 1997 study, there was no increase in Hyp with eccentric exercise up to 9 days post exercise, while the concentric protocol also had no effect. Neither protocol altered total ALP concentrations over the same period.

2.6.2.2 Studies published since 2000

In a follow-up study, Langberg *et al.* measured P1CP and 1CTP each day for 6 days following a competitive marathon (Langberg *et al.*, 2000). A transient but significant reduction in P1CP was seen 1 h after the race followed by a significant increase above pre-exercise values at 72 h post-exercise. In contrast, there was a significant increase in 1CTP at 1 h post-exercise but no further changes thereafter. In the same year, Wallace *et al.* examined changes in 1CTP, P1CP, bone ALP and OC to a 30 min, incremental bout of cycling exercise that concluded with 20 min at ~80% VO_2max (Wallace *et al.*, 2000). There was no effect of exercise on OC, but P1CP and bone ALP were transiently increased immediately after exercise. 1CTP was also increased at the end of exercise and maintained at 90 min post-exercise.

Table 2.6. Studies published since 2000 that measured changes in bone turnover markers in response to acute exercise

Study	Subjects	Age (mean)	Status/Activity	Mode	Duration/Intensity	FAST[1]	CON[2]	PV[3]	Findings
Langberg *et al.* (2000)	m (17)	31	VO_{2max} = 60	RUN	Marathon 3:34 (2:47 - 4:21)	N	N	N	P1CP ↓ (0 h); ↑ (3 d) 1CTP ↑ (0 h)
Rudberg *et al.* (2000)	f (7)	23	Some regular exercise	RUN	30-40-min jogging just below subjective LT (96-148 bpm)	2 h	N	Y	1CTP ↔ (0 to 20 min) OC ↔ (0 to 20 min) total ALP ↔ (0 to 20 min) bone ALP (isoform B2) ↑ (0 min)
Wallace *et al.* (2000)	m (17)	26	VO_{2max} = 56	CYC	5 min @ 1watt/kg, 5 min at 20-min @ 80% VO_{2max}	3 h	Y	Y	OC ↔ bone ALP ↑ vs CON (0 h) P1CP ↑ vs CON (0 h) 1CTP ↑ 7% (0 h to 2 h)
Zittermann *et al.* (2002)	m (18)	25	17.5 h/wk activity	RUN	30-min @ 60% HR_{max}	Only @ baseline	Y	Y	P1CP ↓ vs CON (3 h) Plasma β-CTX ↔ vs CON
Ehrnborg *et al.* (2003)	m (84) f (35)	25	Elite athletes from Olympic sports	CYC, RUN, ROW, RES	VO_{2max} test	NS	N	N	P1CP ↑ (0 h) ; ↓ (0.5 h to 1.5 h) OC ↔ 1CTP ↑ (0 h) ; ↓ (0.5 h to 2 h)
Heinemeier *et al.* (2003)	m (6)	26	Moderately trained	RUN	60 min @ 12 $km \cdot h^{-1}$ with 3% incline	Y	Y	Y	1CTP ↑ (0 h) P1CP ↓ (6 h)
Guillemant *et al.* (2004)	m (12)	31	VO_{2max} = 62	CYC	60-min @ 80%VO_{2max}	N	N	Y	bone ALP ↔ (0 h to 2 h) plasma β-CTX ↑ (0 h to 2 h)

Study	Subjects	Age (mean)	Status/Activity	Mode	Duration/Intensity	FAST[1]	CON[2]	PV[3]	Findings
Whipple *et al.* (2004)	m (9)	22	Untrained	RES	3 x 10 reps of 7 exercises at 10 RM	NS	Y	N	bone ALP ↑ (0 h) P1CP ↔ (0 h to 2 d) serum NTX ↓ (1 h and 8 h) urinary NTX ↔ (1 d and 2 d)
Maïmoun *et al.* (2005)	m (11) f (10)	73	VO_{2max} = 30.5	RUN	VO_{2max} test	Y	N	N	plasma β-CTX ↔ (0 h) OC ↔ (0 h) bone ALP ↔ (0 h)
Maïmoun *et al.* (2006)	m (7)	24	VO_{2max} = 67.9 W_{max} = 390	CYC	50 min @ 85% (-VT) and 115% (+VT) of VT	Y	N	N	-VT (plasma β-CTX ↔; OC ↔ ; bone ALP ↑ (0 h)) +VT (plasma β-CTX ↑ (0 h); OC ↑ (0 h); bone ALP ↑ (0 h))
Tosun *et al.* (2006)	f (9)	28	Sedentary	WALK	30 min at 60-85% HR_{max} with (WE) and without (E) 5 kg load carried	NS	N	N	OC ↔ (0.25 h) P1NP ↔ (0.25 h) P1CP ↔ (0.25 h) total ALP ↑ in WE (1 d) ; ↓ in W (1 d)
Herrmann *et al.* (2007)	m (8)	26	Athlete	CYC	60-min @ 75%, 95% and 110% IAT	NS	N	N	75% (plasma β-CTX ↓ ; TRACP ↔; OC ↓; P1NP ↓); 95% (plasma β-CTX ↑ ; TRACP ↔; OC ↑; P1NP ↔) ; 110% (plasma β-CTX ↑ ; TRACP ↔; OC ↑; P1NP ↑)
	m (7)	36	Sedentary						75% (plasma β-CTX ↓ ; TRACP ↔; OC ↓; P1NP ↓); 95% (plasma β-CTX ↔ ; TRACP ↑; OC ↑; P1NP ↓) ; 110% (plasma β-CTX ↑ ; TRACP ↔; OC ↔; P1NP ↑)
	f (8)	24	Athlete						75% (plasma CTX ↓ ; TRACP ↔; OC ↓; P1NP ↓); 95% (plasma β-CTX ↔ ; TRACP ↔; OC ↑; P1NP ↔) ; 110% (plasma β-CTX ↔ ; TRACP ↑; OC ↔; P1NP ↔)
	f (9)	25	Sedentary						75% (plasma β-CTX ↔ ; TRACP ↔; OC ↓; P1NP ↓); 95% (plasma β-CTX ↑ ; TRACP ↔; OC ↑; P1NP ↓) ; 110% (plasma β-CTX ↔ ; TRACP ↔; OC ↑; P1NP ↔)

Study	Subjects	Age (mean)	Status/Activity	Mode	Duration/Intensity	FAST[1]	CON[2]	PV[3]	Findings
Mouzopoulos *et al.* (2007)	m (16)	32	Athletes	RUN	245 km (32 – 40 h)	Y	N	N	1CTP ↔ Hyp ↓ (0 h), ↑ (1 d, 3 d and 5 d) OC ↓ (0 h and 1 d) P1CP ↓ (0 h) bone ALP ↓ (0 h and 1 d)
Olesen *et al.* (2007)	m (6)	29	Experienced marathon runners	RUN	36 km @ 12 km·h^{-1}	Y	Y	Y	1CTP ↔ (0 h – 4 d) P1CP ↔ (0 h – 4 d)
Pomerants *et al.* (2008)	m (30)	13	VO_{2max} = 39.4	CYC	30 min, 95% VT	N	N	N	1CTP ↔ (0 h and 0.5 h) P1NP ↔ (0 h and 0.5 h)
Maïmoun *et al.* (2009)	m (10) f (8)	72	VO_{2max} = 32	WALK	VO_{2max} Test	Y	N	N	plasma β-CTX ↔ (0 h); OC ↔ (0 h); bone ALP ↔ (0 h)
	m (10) f (8)	72	VO_{2max} = 27						plasma β-CTX ↔ (0 h); OC ↔ (0 h); bone ALP ↔ (0 h)
	m (5) F (4)	26	VO_{2max} = 59						plasma β-CTX ↔ (0 h); OC ↔ (0 h); bone ALP ↔ (0 h)

Study	Subjects	Age (mean)	Status/Activity	Mode	Duration/Intensity	FAST[1]	CON[2]	PV[3]	Findings
Kerschan-Schindl *et al.* (2009)	m (16) f (2)	43	Healthy	RUN	246 km (32 – 35 h)	NS	N	N	plasma β-CTX ↑ (0 h and 3 d) OC ↓ (0 h), ↔ (3 d)

[1] were subjects fasted overnight prior to exercise? (Y, yes; N, no; NS, not stated; 2 h, for 2 h pre-exercise); [2] was a control group included?; [3] were bone markers concentrations corrected for changes in plasma volume (PV)?; ↑, increase; ↓ decrease; ↔, unchanged; 1CTP, c-terminal cross-linking telopeptides of type 1 collagen generated by matrix metalloproteinases; ALP, alkaline phosphatase; β-CTX, c-terminal telopeptide of type 1 collagen; bpm, beats per min (heart rate); CON, control group; CYC, cycling; f, female; HR, heart rate;HR_{max}, heart rate maximum; HYP, hydroxyproline; IAT, individual anaerobic threshold; LT, lactate threshold; m, male; NTX, n-terminal telopeptide of type 1 collagen; OC, osteocalcin; P1CP, c-terminal propeptides of type 1 procollagen; P1NP, n-terminal propeptides of type 1 procollagen; RES, resistance RM, repetition maximum; ROW, rowing; RUN, running; TRACP, tartrate-resistant acid phosphatise; VO_{2max}, maximum oxygen uptake ($ml \cdot kg^{-1} \cdot min^{-1}$); VT, ventilatory threshold; WALK, walking; W_{max}, workload maximum.

Rudberg *et al.* (2000) examined the effect of cycling and jogging on different isoforms of bone ALP as well as 1CTP and OC. Neither an incremental cycling test to exhaustion lasting ~30 min or a 5 km run had any effect on 1CTP or OC when measured immediately and 20 min after exercise. Total ALP was increased immediately after exercise with cycling as was isoform B/I, B1 and B2. In contrast there was no effect of jogging on total ALP and only B2 was increased by exercise.

The first study to examine changes in plasma β-CTX was published in 2002 (Zittermann *et al.*, 2002). In this study, male athletes completed 60 min of running exercise at a speed corresponding to 70% of their running speed at a blood lactate concentration of 4 $mmol \cdot L^{-1}$. Exercise was preceded by a fasted blood sample at 0830 h followed by a standardised breakfast. At 3 h post-exercise (1330 h), β-CTX concentrations had declined significantly and were not different compared to a rested control condition. In contrast, P1CP significantly decreased with exercise but not during the resting condition suggesting the suppression of P1CP formation by exercise.

Further studies of 1CTP and P1CP were published in 2003. Ehrnborg *et al.* observed significant increases in 1CTP and P1CP immediately after a range of different maximal exercise tests (rowing, cycling running) with concentrations falling below pre-exercise values in the first 2 h of recovery (Ehrnborg *et al.*, 2003). Heinemeier *et al.* (2003) report a transient increase in 1CTP immediately after a standardised 60 min run at 12 $km \cdot h^{-1}$ but no further changes up to 68 h post-exercise. P1CP was transiently decreased at 6 h post-exercise, but again, concentrations were not different from baseline thereafter.

In 2004, Guillemant *et al.* published the first paper to show the time course of changes in plasma β-CTX with acute exercise (Guillemant *et al.*, 2004). Their measurements made during and for 2 h after 60 min of cycling exercise at 80% VO_{2max} showed that, in subjects who had eaten a standardised breakfast beforehand, β-CTX concentrations begin to increase during exercise but the majority of the increase occurs in the immediate post-exercise period. Peak concentrations (+45% from pre-exercise) were seen at about 1 h after the termination of exercise. Across an identical time course, they saw no change in bone ALP concentrations. The first and only study to examine changes in both serum and urinary NTX with acute exercise was made in 2004 by Whipple *et al.* who studied an acute bout of moderate resistance exercise (Whipple *et al.*, 2004). They observed a significant reduction in serum NTX at 8 h post-exercise that was no longer present at 24 h post-exercise. There was no change in bone ALP or P1CP at 8 h post exercise resulting in a significant increase in the ratio of markers of bone formation to those of bone resorption.

In 2005, Maïmoun *et al.* published the first study to examine changes in plasma β-CTX in older (60-88 y) adults. They found no change in β-CTX concentrations immediately following an incremental maximal exercise test. There were also no effects on OC or bone ALP concentrations at the same time point although no subsequent measures were taken (Maïmoun *et al.*, 2005).

In 2006, Tosun *et al.* published what appears to be the first paper to measure changes in P1NP with acute exercise and to date, the only paper to measure both P1CP and P1NP in the same study (Tosun *et al.*, 2006). In this study healthy young women performed 30 min of brisk walking with and without a 5 kg backpack. Blood samples taken immediately and 15 min after exercise showed no changes in P1CP, P1NP, 1CTP or OC. The only marker to change was total ALP which, at 24 h post-exercise, was decreased with walking but increased by walking with a backpack. The authors choose not to statistically analyse creatinine-corrected fDPD measured in spot samples taken before and 24 h post-exercise as 'standard deviations were too high' (Tosun *et al.*, 2006).

In the same year, Maïmoun *et al.* (2006) examined changes in bone turnover markers with 50 min of cycling at two different intensities, 85% and 115% of the ventilatory threshold (VT). In this well controlled study, subjects were fasted overnight before the exercise test and all tests began between 0800 h and 0830 h. They showed that immediately after exercise, OC and plasma β-CTX increased only at 115% of VT. In contrast, bone ALP increased by an equal amount at both intensities. Although all markers had returned to baseline values by 15 min post-exercise, this study provided the first direct evidence that the response of bone turnover markers to acute exercise might, in part, be determined by exercise intensity.

A year later, Herrmann *et al.* (2007) also looked at the effects of increasing exercise intensity on changes in plasma β-CTX, TRACP, P1NP and OC. This is the first study to measure the TRACP 5b response to acute exercise and the first study to measure P1NP with high intensity exercise. In a complicated design, male and female athletes and male and female sedentary controls cycled for 60 min at 75%, 95% and 110% of anaerobic threshold (AT). TRACP 5b increased at 3 h and 24 h post-exercise in male controls and female athletes but showed no other changes. In the 75% AT condition, β-CTX decreased in all groups except female controls, whereas at 95% of AT it was increased at 3 h post-exercise in male athletes and female controls. At 110% of AT it was increased at 3 h and 24 h in male athletes and controls only. OC decreased in all groups at 75%, increased in all groups at 95%, and increased in male athletes and female controls only at 110% of AT. Like OC, P1NP decreased in all groups at 75% of AT, in male and female controls only at 95% of AT, and increased at 3 h and 24 h in male controls and male athletes respectively. This study, however, did not standardise either the time of day of the exercise tests or the nutritional status (*i.e.* fasted or fed) of subjects, two factors known to influence marker levels. Although this study shows a degree of heterogeneity in responses at the same exercise intensity in the four different groups, like Maïmoun *et al.* (2006) the data also indicates an effect of increasing exercise intensity on changes in bone turnover markers with acute exercise.

Using an identical protocol to that of Langberg *et al.* (1999), Olesen *et al.* measured 1CTP and P1CP immediately after and at 24 h, 48 h, 72 h and 96 h after a 36 km run at 12 $km \cdot h^{-1}$ (Olesen *et al.*, 2007). Like Langberg *et al.* (1999) they saw no change in serum 1CTP with exercise, but unlike Langberg *et al.* they also observed no effect of exercise on P1CP. The first study of an ultra endurance race on BTM was made by Mouzopoulos *et al.* who measured Hyp, 1CTP, P1CP, OC and bone ALP in the hours and days following the Spartathlon, a 245 km running race. They observed no change in 1CTP but significant reductions in Hyp and all bone formation markers in the post-exercise period (Mouzopoulos *et al.*, 2007).

In 2008, Pomerants *et al.* examined changes in 1CTP and P1NP in response to 30 min of cycling exercise at 95% of VT in adolescent boys (Pomerants *et al.*, 2008). They found no change in either marker when measured immediately after exercise or after 30 min of recovery.

In 2009, Maïmoun *et al.* performed a follow-up study to their 2005 investigation to determine if the bone marker response to an incremental maximal exercise test was affected by both age and fitness level (Maïmoun *et al.*, 2009). Highly active elderly subjects were compared to moderately active subjects of a similar age as well as young, active subjects. As in their 2005 study, they found no effect of maximal exercise on plasma β-CTX, OC or bone ALP in highly active elderly group and the same was true for the active, young group. The only change they observed was a significant increase in bone ALP immediately after exercise in the less active elderly group.

Like Mouzopoulos *et al.* (2007), the most recent study of changes in BTM with acute exercise also studied the Spartathlon but measured plasma β-CTX rather than 1CTP (Kerschan-Schindl *et al.*, 2009). They report a ~65% increase in β-CTX immediately after the race that remained 40% above pre-race values the morning after the race. OC concentrations were suppressed ~50% immediately after the race although, unlike β-CTX, they had returned to pre-race levels the morning after the race. In this study is was possible to partially standardise the time of day (morning) of the pre-race and the second post-race blood samples but as subjects completed the race in ~32-35 h this was not possible for the immediate post-race sample which were collected in the mid-afternoon and early evening. Also, they do not report standardising food intake prior to any of the sampling points.

2.6.2.3 *Possible influence of mechanical loading on findings from studies examining the effects of exercise on bone turnover markers*

As highlighted in section 2.6.1.1, in studies of acute exercise in humans, it is not possible to differentiate mechanical effects on bone from those resulting from metabolic and/or endocrine changes. Therefore, it must be assumed that changes in circulating bone turnover markers reflect the summation of these all these effects and possibly others as well. If the aim of an investigation is simply to examine the effect of 'exercise' on bone turnover markers, or to compare different types of exercise (*e.g.* Rong *et al.*, 1997; Ehrnborg *et al.*, 2003), investigators much acknowledge this

limitation in their ability to identify the individual contribution of these different mechanisms to their findings. However, in studies that have deliberated manipulated a 'traditional' cardiorespiratory variable such as cardiovascular intensity or exercise duration (*e.g.* Maïmoun *et al.*, 2006; Herrmann *et al.*, 2007) in order to examine its impact on changes in bone turnover markers, further consideration must be paid to the influence of mechanical loading on findings. For example, in studies where cardiovascular exercise intensity has been varied during cycling (Maïmoun *et al.*, 2006; Herrmann *et al.*, 2007), it is highly likely that mechanical loads acting on bone generated from muscular forces will increase as muscle activity increases as a function of cardiovascular intensity. Likewise, although no study to date has manipulated cardiovascular exercise intensity during acute, weight-bearing exercise, as cardiovascular intensity increases so will muscular forces and GRFs resulting in increased strain magnitudes and strain rates (Burr *et al.*, 1996). There will also be an increase in the frequency of impacts and thus total number of loads. Similarly, although no published study has varied exercise duration, increasing duration not only increases the metabolic demand of exercise but also the number of times that mechanical loads are applied to bone. Therefore, with studies of this nature it is difficult to establish to what extent differences in changes in bone turnover markers can be attributed to variation in the cardiorespiratory variable itself.

One interesting finding from published studies that have varied cardiovascular exercise intensity (Maïmoun *et al.*, 2006; Herrmann *et al.*, 2007) is that increases in bone turnover markers, particularly β-CTX, only occur at higher exercise intensities. Although this might be related to endocrine effects of PTH (see section 2.6.3.4 below), as high stresses can result in damage to bone and an acceleration in bone remodelling is accelerated (Bentolila *et al.*, 1998; Mori and Burr, 1993), it is also possible that changes in bone turnover markers reflect processes associated with damage. However, in animals model damage and remodelling are associated with bone fatigue (Herman *et al.*, 2010) and, as it is not possible to measure bone fatigue or confirm the occurrence of damage in humans during acute, exercise, this possibility remains only speculative.

2.6.2.4 Summary

The studies reviewed in this section indicate that a single bout of acute exercise is associated with alterations in BTMs. However, whilst the volume of literature in this field has expanded in the last 20 years, it remains relatively understudied compared to other areas of bone biology. Drawing firm conclusions regarding the effects of acute exercise on BTM from this relatively small number of studies is severely hampered by the wide variation in study design in terms of the subjects studied, the exercise protocol used and the timings BTM measurements. Equally importantly, the changing availability and continuing improvement in the specificity of BTM for the resorption and formation processes confounds the comparison of studies that are otherwise similar in design.

As can be seen in Table 2.7, only since 2002 have studies measured serum markers that are considered be the most specific for the individual processes of bone resorption (*e.g.* plasma β-CTX) and formation (*e.g.* P1NP, bone ALP) and these studies have examined a variety of different modes of exercise from walking to high intensity cycling and resistance exercise. Only seven studies have examined plasma β-CTX, considered one of the more specific markers of bone resorption, and again no more than three of these have examined the same mode of exercise (endurance cycling).

Table 2.7. Summary of key features of published studies that have measured changes in specific bone turnover markers in response to acute exercise.

Criteria	# of Studies	Comments / Strengths / Weaknesses
Measured specific markers of resorption (plasma β-CTX, NTX, DPD) *and* formation (P1NP, bone ALP)	7	Three studies have examined cycling (Guillemant *et al.*, 2004; Maïmoun *et al.*, 2006; Herrmann *et al.*, 2007), two studies examined VO_{2max} tests (Maïmoun *et al.*, 2005, 2009), one study has examined walking (Tosun *et al.*, 2006) and one study has examined resistance exercise (Whipple *et al.*, 2004). No studies have examined endurance running.
Measured plasma β-CTX as a marker of bone resorption	7	Two studies have examined VO_{2max} tests (Maïmoun *et al.*, 2005, 2008), three have examined endurance cycling (Guillemant *et al.*, 2004; Maïmoun *et al.*, 2006; Herrmann *et al.*, 2007) and one has examined ultraendurance running (Kerschan-Schindl *et al.*, 2009). Only one study examined endurance running (Zittermann *et al.*, 2002).
Measured plasma β-CTX as a marker of bone resorption in response to endurance exercise	3	Acute plasma β-CTX response to endurance cycling unclear: transient increase during exercise only in fasted subjects (Maïmoun *et al.*, 2006) or majority of increase in 1st hour post-exercise in pre-fed subjects (Guillemant *et al.*, 2004). Elevated at 3 h and 24 h post exercise (Herrmann *et al.*, 2007); β-CTX increased only with high intensity cycling (Maïmoun *et al.*, 2006; Herrmann *et al.*, 2007). β-CTX decreased 3 h after endurance running (Zittermann *et al.*, 2002).
Measured plasma β-CTX as a marker of bone resorption in response to endurance running	1	Zittermann *et al.* (2002) examined relatively low intensity exercise in well-trained runners, subjects were all non-fasted prior to exercise, only measured P1CP as a marker of bone formation, and post-exercise measures taken only at 3 h of recovery. Kerschan-Schindl *et al.* (2009) examined ultraendurance running but did not standardise subject's acute nutritional status or tightly control the time of day of blood samples.
Measured P1NP as a marker of bone formation	3	Tosun *et al.* (2006) examined walking exercise and Pomerants *et al.* (2008) examined cycling exercise but only measured P1NP up to immediately post-exercise; Herrmann *et al.* (2007) examined cycling and did not standardise time of day or subject acute nutritional status.

Criteria	# of Studies	Comments / Strengths / Weaknesses
Measured specific resorption and formation marker response to acute exercise at 24 h	2	Tosun *et al.* (2006) examined walking exercise up to 24 h post-exercise but did not standardise subject's acute nutritional status or statistically analyse DPD/Cr; Herrmann *et al.* (2007) examined cycling up to 24 h post-exercise but did not standardise acute nutritional status or the time of day of exercise.
Measured specific resorption and formation marker responses to acute exercise beyond 24 h	1	Kerschan-Schindl *et al.* (2009) examined ultraendurance running but did not standardise subject's acute nutritional status or tightly control the time of day of blood samples, and did not measure a specific marker of bone formation – studies of less specific markers (P1CP and 1CTP) suggest changes in bone turnover with acute exercise beyond 24 h (Langberg *et al.*, 1999; Langberg *et al.*, 2000).

1CTP, c-terminal cross-linking telopeptides of type 1 collagen generated by matrix metalloproteinases; ALP, bone alkaline phosphatise; β-CTX, c-terminal telopeptide of type 1 collagen; DPD/Cr, creatinine-corrected deoxypyridinoline, NTX, n-terminal telopeptide of type 1 collagen; P1CP, c-terminal propeptides of type 1 procollagen; P1NP, n-terminal propeptides of type 1 procollagen; VO_{2max}, maximum oxygen uptake.

Despite the similar mode of exercise, the acute response of plasma β-CTX remains unclear, with a transient increase during exercise only (Maïmoun *et al.*, 2006) or the majority of the increase in first 90 min post-exercise (Guillemant *et al.*, 2004) that possibly lasts up 24 h post exercise (Herrmann *et al.*, 2007) being reported. Together these studies also suggest that plasma β-CTX concentrations might only increase with high intensity exercise (Maïmoun *et al.*, 2006; Herrmann *et al.*, 2007).

These studies also differ in the nutritional state of subjects prior to exercise and one (Herrmann *et al.*, 2007) did not standardise nutritional status or the time of day at which exercise was performed. As plasma β-CTX has a marked circadian rhythm, the amplitude of which is enhanced by feeding, both time-of-day and pre-exercise nutritional state might also influence its response to acute exercise but to date this has not been explored. Only one study has examined the plasma β-CTX response to endurance running (Zittermann *et al.*, 2002). This study examined relatively low intensity exercise, used well-trained subjects who were non-fasted prior to exercise, measured P1CP as a marker of bone formation, and only took one post-exercise measure at 3 h of recovery. There is, therefore, much to be learnt regarding the BTM response to endurance running.

Despite its preferred use in clinical studies as a bone formation marker, there is scant information regarding changes in P1NP with acute exercise (Table 2.6.3). As P1NP reflects an early stage of the bone formation process and has a rapid and marked response to anabolic activity (Glover *et al.*, 2009), useful information regarding the effect of acute exercise on bone turnover might be gained by examining changes in its concentrations.

Finally, despite some of the early studies that showed changes in less specific BTM several days after a bout of acute exercise (*e.g.* Langberg *et al.*, 2000), there exists little information regarding changes in specific BTM on the days that follow an acute bout of exercise and no information on endurance running (Table 2.7). As both endurance athletes and military recruits – two groups in which physical activity is sometimes associated with deleterious changes in bone – frequently perform exercise on consecutive days during periods of training, it is of interest to better understand if the early changes in specific BTM reported in some previous studies persist on subsequent days, or if there are delayed fluctuations in their levels in response to exercise. Measurement of bone formation markers during this period may be of particular relevance as any change in their levels would be expected to occur subsequent to any changes in bone resorption markers.

2.6.3 The PTH response to acute exercise

The PTH response to acute exercise has received considerable attention with studies utilising protocols ranging from supramaximal exercise through to walking. A summary of these studies is provided in Table 2.8.

2.6.3.1 The PTH response to supramaximal exercise

No significant change in PTH is reported at either 5 min or 60 min after a supramaximal Wingate test (Kristofersson *et al.*, 1995). Similarly, exhaustive, high intensity running lasting 1 to 2 min also does not affect PTH concentrations in the first 90 min of recovery from exercise (Cunningham *et al.*, 1985). PTH concentrations are unchanged immediately after a 2 min maximal concentric leg exercise protocol but increase in the first 5 min post-exercise (Joborn *et al.*, 1988). Two min of maximal, isokinetic leg exercise has no effect on PTH during exercise but is transiently increased at 30 min post-exercise (Ljunghall *et al.*, 1985).

2.6.3.2 The PTH response to maximal exercise

O'Neil *et al.* (1990) report unchanged PTH concentrations in response to a VO_{2max} test. In contrast, later studies showed a significant increase in PTH concentrations immediately after a VO_{2max} test where concentrations were increased ~25 to 35% in elderly men and women (Maïmoun *et al.*, 2005; Maïmoun *et al.*, 2009). In younger subjects, this increase was ~50% in response to the same protocol (Maïmoun *et al.*, 2009). In elderly men, before and after 6 weeks of endurance training, PTH is increased 11% and 22% immediately following a cycling VO_{2max} test (Zerath *et al.*, 1997).

Table 2.8. Changes in parathyroid hormone (PTH) with acute exercise.

Reference	Exercise Protocol			PTH
	Type	*Protocol*	*Duration*	
Supramaximal				
Cunningham *et al.* (1985)	RUN	Maximum effort until exhaustion	60-130 sec	↔
Ljunghall *et al.* (1985)	RES	Maximum effort, isokinetic knee flexion and extensions	2 min	
During exercise				↔
@ 30 min post-exercise				↑
Kristofersson *et al.* (1995)	CYC	Max effort	30 sec	↔
Maximal				
O'Neill *et al.* (1990)	RUN	INC to exhaustion (VO_{2max} test)	14.1 ± 2.2 min	↔
Zerath *et al.* (1997)	CYC	INC to exhaustion (VO_{2max} test)	NS	
Before 6 wk endurance training				↑
After 6 wk endurance training				↑
Maïmoun *et al.* (2005)	WALK	INC to exhaustion (VO_{2max} test)	8-12 min	↑
Maïmoun *et al.* (2009)	WALK	INC to exhaustion (VO_{2max} test)	8-12 min	↑
Non-steady-state endurance				
Ljunghall *et al.* (1984b)	CYC	INC to exhaustion	NS	↑
Aloia *et al.* (1985)	RUN	INC (40-75% VO_{2max})	20 min	↓
Henderson *et al.* (1989)	CYC	INT, INC (40-90% VO_{2max})	20 min	↑
Salvesen *et al.* (1994)	RUN			
male endurance runners		INC (141-177 bpm)	40 min	↑
Brahm *et al.* (1997a)	RUN	INC (47-100% VO_{2max})	35 min	↑
Brahm *et al.* (1997b)	RES	One-legged knee extension (38-100 W_{max})	30 min	↔
Takada *et al.* (1998)	CYC	INC (maximum anaerobic power)	NS	
@ 0 h post exercise				↓
@ 30 min post-exercise				↑
Rudberg *et al.* (2000)	CYC	INC to exhaustion	(20-32) min	↔
Submaximal endurance				
Ljunghall *et al.* (1984b)	CYC	SS (65% W_{max})	60 min	↔
	CYC	300 W to exhaustion	3.5-6.5 min	↔

Reference	Exercise Protocol			PTH
	Type	*Protocol*	*Duration*	
Ljunghall *et al.* (1986)	CYC	SS (50 % W_{max})	5 h	↑
Nishiyama *et al.* (1988)	RUN	SS (43-52% of 'maximum effort')	30 min	↑
Grimston *et al.* (1993)	RUN	SS	45 min	
normal BMD				↓
low BMD				↑
Salvesen *et al.* (1994)	RUN		50 min	
male endurance runners		SS (157-169 bpm)		↑
male fire fighters		SS (143-156 bpm)		↔
Thorsen *et al.* (1996)	WALK	SS (50% VO_{2max})	90 min	↔
Rong *et al.* (1997)	CYC	SS (85% VO_{2max})	15 min	↔
		SS (55% VO_{2max})	45 min	↔
Tsai *et al.* (1997)	CYC	SS (60% VO_{2max})	60 min	↑
Thorsen *et al.* (1997)	RUN	SS (50% of VO_{2max})	45 min	
@ 1 h post-ex				↔
@ 24 h post-ex				↑
@ 72 h post-ex				↑
Rudberg *et al.* (2000)	CYC	SS (96–148 bpm)	30-40 min	↑
Zittermann *et al.* (2002)		70% of speed at 4 mmol BL	60 min	↔
Bouassida *et al.* (2003)	RUN	SS (21 min @ 70%, 21 min @ 85%	42 min	
@ 0 h post-ex		VO_{2max})		↑
@ 24 h post-ex				↑
Guillemant *et al.* (2004)	CYC	SS (80% VO_{2max})	60 min	↑
Tosun *et al.* (2006)	WALK	SS (60–85% HR_{max})	30 min	
with a 5k back pack				↑
without a 5k back pack				↑
Maïmoun *et al.* (2006)	CYC	SS (85% or 115% VT)	50 min	
@ 85% VT				↔
@115% VT				↑
Barry and Kohrt, (2007)	CYC	SS (60-75% VT)	2 h	
without PV correction				↑
with PV correction				↑
Mouzopoulos *et al.* (2007)	RUN	INT (245 km)	32.3-39.7 h (breaks, 3.26-4.82 h)	↑

Reference	Exercise Protocol			PTH
	Type	*Protocol*	*Duration*	
Resistance exercise				
Rong *et al.* (1997)	RES	5 x 8 reps (85% of 3 RM)		↑

PTH, parathyroid hormone; ↑, increase; ↓ decrease; ↔, unchanged; bpm, beats per min (heart rate); BL, blood lactate; CYC, cycling; HR_{max}, maximum heart rate; NS, not stated; PV, plasma volume; RES, resistance exercise; RM, repetition maximum; RUN, running; SS steady-state; INC, incremental; INT, intermittent; VO_{2max}, maximum oxygen uptake; VT, ventilatory threshold; WALK, walking; W_{max}, workload max.

2.6.3.3 The PTH response to non-steady state, endurance exercise

Several studies examined changes in PTH using longer, non-steady state protocols. Ljunghall *et al.* (1984b) report no effect of an incremental cycling to exhaustion on PTH concentrations. The following year, Aloia *et al.* (1985) report a significant decrease in PTH concentrations in the early part of four stage, 20 min cycling protocol in which the intensity was increased from 40% to 75% VO_{2max}. In the study of Henderson *et al.* (1989) where subjects cycled at progressively increasing loads at 50%, 60%, 70% and 90% of VO_{2max} separated by 2 min of active recovery at 25% VO_{2max}, PTH increased during exercise at 70% and 90% of VO_{2max} only. Peak concentrations (+86%) occurred at 15 min post-exercise but were no longer elevated at 45 min post-exercise. Similarly, Salveson *et al.* (1994) observed a 50-60% increase in PTH only in the final two stages of a five stage, 40 min incremental run ending at ~86% of age-predicted maximum heart rate. Brahm *et al.* (1997a) had subjects perform a 35 min incremental treadmill run ranging from 30% to 76% VO_{2max} and finishing with 5 min at maximal effort. PTH was increased (~20%) and remained elevated at 30 min and 24 h post-exercise. In contrast, a similar incremental protocol that used one legged knee-extension exercise had no effect on PTH concentrations (Brahm *et al.* 1997b). Subjects in the study of Rudberg *et al.* (2000) performed incremental exercise (30 W every 6 min) to exhaustion lasting 20 to 32 min and, like Brahm *et al.* (1997b) observed no increase in PTH measured immediately after exercise. Takada *et al.* (1998) report a suppression of PTH immediately after a maximal anaerobic power test but subsequently concentrations were increased in the first 30 min post-exercise.

2.6.3.4 The PTH response to steady state, endurance exercise

The majority of the studies of changes in PTH with acute exercise have used steady-state protocols with a wide variety of exercise durations and intensities. Ljunghall *et al.*, (1984b) report no change in PTH with cycling at 65% W_{max} for 60 min or at 300 W until exhaustion. Two years later, using a 5 h bout of cycling exercise at 40-50% VO_{2max}, Ljunghall *et al.* (1986) observed a significant increase in PTH that was evident throughout and at the end of exercise. Nishiyama *et al.* (1988) report a ~33% increase in PTH immediately after a 30 min run at ~50% effort with concentrations returning to

pre-exercise levels by 1 h post-exercise. Grimston *et al.* (1993) report that 45 min running at 'training pace' had no effect on PTH concentrations either immediately after exercise or at 45 min post-exercise in trained female runners with normal bone mineral density although PTH was increased in those with low BMD. Salvesen *et al.* (1994) report no change in PTH in trained runners during a 50 min run at 4.2 $m \cdot s^{-1}$. In contrast, they report a 40% increase in PTH in trained fire fighters after only 10 min of a 50 min run at 3·3 $m.s^{-1}$, which increased to 60% by the end of exercise.

Brahm *et al.* (1996) measured PTH at 1 and 2 days after a running race in which male and female subjects ran 15 (range 5-30) and 28 (range 15-30) km at a mean speed of 5:30 and 5:02 $min \cdot km^{-1}$. They observed no changes in PTH concentrations from pre-race levels on either of the two recovery days. Likewise, Thorsen *et al.* (1996) report no effect of 90 min walking at 50% VO_{2max} on PTH. Rong *et al.* (1997) compared the PTH response to cycling at 45 min at 55% of VO_{2max} and 15 min at 85% of VO_{2max}. They observed a significant decline in PTH immediately after and at 1 h after both forms of exercise compared with pre-exercise levels. This pattern of change was identical to that of a resting control group indicating no effect of exercise on PTH. In contrast, Tsai *et al.* (1997) report a 50% increase in PTH immediately after 60 min of cycling at 60% VO_{2max}.

No effect of exercise on PTH concentrations was seen at 1 h post-exercise in young women jogging for 45 min at 50% of VO_{2max} (Thorsen *et al.*, 1997). However, PTH increased ~60% in blood samples drawn at 24 h and 72 h post-exercise making this one of the only studies to show a prolonged effect of exercise on PTH. In a similar group of subjects, 30–40 min jogging at an '*even pace just below their subjective lactate accumulating effort level*' was associated with an ~50% increase in PTH immediately after exercise with concentrations returning to pre-exercise 20 min later (Rudberg *et al.*, 2000). No PTH response was observed in male athletes 3 h after a 60 min run at an intensity described as '*70% of the speed at 4 mmo·L^{-1} blood lactate*' (Zittermann *et al.*, 2002).

In young, active men, PTH concentrations are increased 10 to 15% after only 7 min of running at 70% VO_{2max} and the increase maintained after 21 min of exercise (Bouassida *et al.*, 2003). In this study subjects continued to exercise for a further 21 min at 85% VO_{2max} and by the end of exercise PTH concentrations were ~80% higher than pre-exercise levels and remained ~5% elevated at 24 h post-exercise. Interestingly, when exercise at 70% VO_{2max} and 85% VO_{2max} were separated by a 40 min rest break, the increase in PTH with exercise at 70% was maintained, but no further increase is seen at the end of exercise at 85% and concentrations at 24 h post-exercise were not different from pre-exercise levels. Guillemant *et al.* (2004) observed a 2.5 to 3-fold increase in PTH in response to 60 min of cycling at 80% VO_{2max} that rapidly returned to baseline in the first hour post-exercise. When subjects consumed high calcium mineral water before and during exercise, PTH was again increased but the magnitude of the increase was attenuated, with post-exercise concentrations only 70% above pre-exercise levels. By measuring PTH before, during and in the hours following, thus

study demonstrates a transient increase in PTH with endurance exercise characterised by a prompt rise with the onset of exercise and a rapid return to pre-exercise levels with its termination.

In young women, 30 min of brisk walking at a intensity of 60 to 85% of HR_{max} results in a ~100% increase in PTH concentrations immediately after exercise, than remains partially elevated at 15 min post-exercise (Tosun *et al.*, 2006). The magnitude of this response was similar when subjects walked wearing a 5 kg backpack.

Maïmoun *et al.* (2006) report no increase in PTH during or at 15 min after 50 min of cycling at 85% VT, whereas at 115% VT, PTH was increased ~40% by the end of exercise and by 80% at 15 min post-exercise. In contrast, however, 2 h of cycling at only 60 to 75% of VT also increased PTH by ~80% immediately after exercise (Barry and Kohrt, 2007). Finally, in the only study of ultra-endurance exercise, PTH is increased by ~33% immediately after but not 1 day after a 245 km running race lasting 32-35 h (Mouzopoulos *et al.*, 2007).

In summary, this section has shown that acute exercise, particularly endurance-type exercise, is a potent stimulator of circulating PTH concentrations. Although short-term, supramaximal exercise is not associated with an increase in PTH, there is some evidence from short-term, well controlled studies that increasing exercise intensity may be an important factor in the PTH response to endurance exercise. Finally, there is conflicting evidence regarding the pattern of change in PTH with acute exercise. Although several studies report increases in PTH at 24 h or more after exercise, other studies suggest that increases in PTH with exercise decreases rapidly with the termination of exercise resulting in only a transient response. Given the differential effects of PTH on bone depending on the time course of changes in its circulating concentrations, a better understanding of the time course of changes in PTH with acute exercise is of considerable importance.

2.6.3.5 Effect of acute exercise on Vitamin D

As PTH stimulates kidney production of $1,25(OH)_2D3$ – the most biologically active metabolite of vitamin D – from the precursor 25(OH)D, and acute exercise appears to result in an increase in PTH, it might be expected that acute exercise would stimulate an increase in $1,25(OH)_2D3$ levels. However, Maïmoun *et al.* (2005) observed an increase in PTH but a significant decrease in 25(OH)D and no change in $1,25(OH)_2D3$ immediately after a VO_{2max} test in active, elderly subjects. More recently, neither 25(OH)D or $1,25(OH)_2D3$ were affected by the same protocol despite increases in PTH (Maïmoun *et al.*, 2009).

2.6.3.6 Effect of acute exercise on calcitonin

Calcitonin (CT) acts to suppress the concentration of serum calcium, opposing some of the actions of PTH. As many studies of acute exercise report increasing serum calcium concentrations, a compensatory increase in CT might be expected. However, the majority of studies report no effect of exercise on CT despite increases in total calcium (Nishiyama *et al.*, 1988; O'Neill *et al.*, 1990) or iCa (Rong *et al.*, 1997; Thorsen *et al.*, 1996; Cunningham *et al.*, 1995). Tosun *et al.* (2006) report no change in CT but measured neither total Ca nor iCa. Two studies do report increases in CT. Aloia *et al.* (1990) observed increased CT concomitant with increases in both total Ca and iCa with 20 min of endurance exercise. Similarly, Grimston *et al.* (1993) report increased total calcium and CT with endurance exercise in female runners with normal BMD. Interestingly, however, in runners with low BMD, exercise also resulted in increased total calcium but CT concentrations were reduced.

2.6.3.7 Effect of acute exercise on parathyroid hormone-related peptide

To date only one study has reported the effects of acute exercise on parathyroid hormone-related peptide (PTHrP) and found no change in its concentrations in response to resistance exercise, or moderate and high intensity cycling (Rong *et al.* 1997). However, PTHrP is unstable in plasma (Pandian *et al.*, 1992) and in clinical studies is frequently undetectable in healthy subjects (Fraser *et al.*, 1993; Strewler, 2000). Therefore, due to the sensitivity of available assays, it may be difficult to detect more subtle changes in serum PTHrP that might occur with exercise.

2.6.4 Relationship between changes in PTH and bone turnover markers in response to acute exercise

Despite its important role in the regulation of bone turnover, capable of inducing both bone resorption and bone formation and associated changes in BTM, the role of increases in systemic PTH during acute exercise on changes in bone turnover remains unknown. Some studies suggest that mechanical loading and PTH can act synergistically to enhance bone formation. For example, Chow *et al.* (1998) showed that a single injection of PTH enhances the anabolic response to loading when given before, but not after, mechanical stimulation. Additionally, the lack of an osteogenic response in thyroparathyroidectomized rats was restored by a single injection of PTH before stimulation, suggesting that physiological levels of PTH are necessary for the mechanical responsiveness of bone. In rats subjected to 3 x weekly PTH injections and bouts of mechanical loading, PTH and loading has synergistic effects on mineral apposition rate, and bone formation rate were observed at the periosteal and endocortical surfaces (Hagino *et al.*, 2001). Similar effects are also reported by Kim *et al.* (2003), who report that, after 4 weeks of stimulation, the combination of PTH and mechanical loading maintained the increase rate of bone formation that had already returned to control levels in animals treated with PTH or mechanical loading alone. Importantly, combining PTH with a loading protocol that alone does not stimulate osteogenesis, can produce osteogenic effects (Sugiyama *et al.*, 2008).

High-dose intermittent PTH combined with remobilisation also induces a greater periosteal bone formation rate and results in a greater cross-sectional area in previously immobilised bones compared with remobilisation or PTH alone (Ma *et al.*, 1999). Although the mechanisms underlining the interaction between the mechanical loading and PTH treatment are not yet completely understood, both a PTH-activated, volume-sensitive calcium influx pathway in osteocytes (Miyauchi *et al.*, 2000) and conformational transitions in the parathyroid hormone type-1 receptor in response to fluid shear stress (Zhang *et al.*, 2009) may be involved.

There are few studies that have measured both PTH and specific BTM (*e.g.* β-CTX, P1NP) responses to acute exercise. This is mainly due to the paucity of well-controlled studies that have assessed changes in specific BTM with acute exercise. Maïmoun *et al.* (2009) and Maïmoun *et al.* (2005) report increases in PTH with VO_{2max} tests but no change in β-CTX immediately after exercise. Exercise increased bone ALP in moderately active elderly subjects, but not in groups of highly active elderly or moderately active young people but, as described previously, no measures were taken in the post-exercise period. Tosun *et al.* (2006) report increased PTH but no change in P1NP with 30 min of walking exercise while Zittermann reports no change in either PTH or β-CTX with a moderate 60 min run (Zittermann *et al.*, 2002).

Maïmoun *et al.* (2006) report no change in PTH or β-CTX with 50 min of exercise at 85% VT although bone ALP was increased during and immediately after exercise. In contrast, exercise at 115% VT increased both PTH and β-CTX, and increased bone ALP by a similar amount as seen with exercise at 85% VT. Although this might suggest a role for PTH in the exercise-associated increase in bone resorption, β-CTX was increased before PTH (30 min *vs* 50 min of exercise). Guillemant *et al.* (2004) showed that a 2.5-3 fold increase in PTH preceded a 45% increase in β-CTX at 1 h post-exercise. Calcium ingestion before and during exercise blunted the increase in PTH to only 70% but completely abolished the increase in β-CTX suggesting that there maybe a threshold for PTH concentrations above which bone resorption is increased. However, unlike Maïmoun *et al.* (2006), in this study bone ALP was unchanged in either condition.

Taken together, these studies suggest that prolonged exposure to increased PTH, such as during endurance rather than brief, high intensity exercise, might be associated with an increase in bone resorption rather than bone formation. However, none of these studies have examined changes in PTH and specific BTM in the days following exercise and, in doing so, might have failed to detect an increase in bone formation markers that would be expected to occur subsequent to an increase in bone resorption. Such an observation would be an important consideration in the interpretation of the role of exercise-associated changes in PTH on bone turnover.

2.6.5 Mechanisms by which acute exercise might increase PTH concentrations

As reviewed in the previous section, the majority of studies of acute exercise have shown that acute exercise results in an increase in circulating PTH concentrations. Based on data from studies of rested humans, several mechanisms have been suggested by which acute exercise might increase PTH concentrations. These include a decrease in total calcium or iCa, an increase in PO_4, alterations in glycaemic status, metabolic acidosis and adrenergic stimulation.

2.6.5.1 Total and ionised calcium

Under resting conditions, the serum iCa concentration controls PTH secretion while simultaneously PTH regulates the serum calcium concentration, so reductions in systemic calcium, both total and ionised, are obvious candidates for the mechanism underlying the exercise-associated increase in PTH. However, there is little consensus on the association between changes in calcium and PTH. A summary of studies that have investigated changes in PTH concomitantly with either total calcium, iCa or both is provided in Table 2.9.

Ljunghall *et al.* (1984b) report increased iCa and PTH with 20 min of incremental exercise, no change in either with 60 min of steady-state exercise at 65% VO_{2max} and increased iCa but unchanged PTH with short-term exhaustive exercise. Aloia *et al.* (1985) showed reduced PTH and increased total calcium and iCa in the early part of a 20 min, incremental exercise test ranging from 40-70% VO_{2max}. Twρ min of maximal, single leg, isokinetic exercise increased total calcium and iCa during exercise without affecting PTH although PTH was increased during early recovery despite both total calcium and iCa being similar to baseline levels (Ljunghall *et al.*, 1985). Cunningham *et al.* (1985) report an increase in total calcium and iCa with 1 to 2 min of exhaustive running but no alteration in PTH. PTH is increased throughout 5 h of exercise at 40 to 50% VO_{2max}, as is total calcium, whilst the iCa concentration is unchanged (Ljunghall *et al.*, 1986). Nishiyama *et al.* (1988) report an increase in PTH and decreases in both total calcium and iCa immediately after a 30 min run at ~50% VO_{2max}. Henderson *et al.* (1989) showed increased total calcium and iCa throughout a 20 min incremental run up to 90% VO_{2max} with PTH increased only in the later stages.

Table 2.9. Changes in parathyroid hormone (PTH) in relation to changes in total serum calcium (tCa) and ionised calcium (iCa) with acute exercise.

Reference	Exercise Protocol			PTH	tCa	iCa
	Type	*Protocol*	*Duration*			
Ljunghall *et al.* (1984b)	CYC	INC to exhaustion	20 min	↑	NM	↑
		SS (65% W_{max})	60 min	↔	NM	↔
		300 W to exhaustion	3.5-6.5 min	↔	NM	↑
Aloia *et al.* (1985)	RUN	INC (40-75% VO_{2max})	20 min	↓	↑	↑
Ljunghall *et al.* (1985)	RES	Max effort isokinetic knee extension	2 min			
During exercise				↔	↑	↑
@ 30 min post-exercise				↑	↔	↔
Cunningham *et al.* (1985)	RUN	Maximum effort until exhaustion	60-130 sec	↔	↑	↑
Ljunghall *et al.* (1986)	CYC	SS (40-50% W_{max})	5 h	↑	↑	↔
Nishiyama *et al.* (1988)	RUN	43-52% of maximum effort	30 min	↑	↓	↓
Henderson *et al.* (1989)	CYC	INT, INC (40-90% VO_{2max})	20 min	↑	↑	↑
Grimston *et al.* (1993)	RUN	SS	45 min			
normal BMD				↓	↑	NM
low BMD				↑	↑	NM
Salvesen *et al.* (1994)	RUN					
Male endurance runners		INC (141-177 bpm)	40 min	↑	↑	NM
male endurance runners		SS (157-169 bpm)	50 min	↑	↑	NM
male firefighters		SS (143-156 bpm)	50 min	↔	↑	NM
Kristofersson *et al.* (1995)	CYC	Max effort	30 sec	↔	↔	↑
Rong *et al.* (1997)	RES	5 sets of eight reps (85% of 3 RM)		↑	NM	↔
	CYC	SS (85% VO_{2max})	15 min	↔	NM	↑
	CYC	SS (55% VO_{2max})	45 min	↔	NM	↔
Thorsen *et al.* (1997)	RUN	SS (50% of VO_{2max})	45 min			
@ 1 h post-ex				↔	NM	↓
@ 24 h post-ex				↑	NM	↔
@ 72 h post-ex				↑	NM	↓
Ashizawa *et al.* (1997)	RES	3 sets of seven exercises (60-80% of 1 RM)	45 min			
@ 0 h post-ex				↔	↑	↓
@ 45 min post-ex				↔	↔	↑
@ 105 min post-ex				↓	↔	↔
Brahm *et al.* (1997a)	RUN	INC (47-100% VO_{2max})	35 min	↑	↑	NM

Reference	Exercise Protocol			PTH	tCa	iCa
	Type	*Protocol*	*Duration*			
Brahm *et al.* (1997b)	RES	One-legged knee extension (38-100 W_{max})	30 min	↔	↔	NM
Tsai *et al.* (1997)	CYC	SS (60% VO_{2max})	60 min	↑	↑	NM
Zerath *et al.* (1997)	CYC	INC to exhaustion (VO_{2max} test)	NS			
Before 6 wk endurance training				↑	↑	NM
After 6 wk endurance training				↑	↑	NM
Rudberg *et al.* (2000)	RUN	SS (96–148 bpm)	30-40 min	↑	↔	↔
	CYC	INC to exhaustion	(20-32) min	↔	↑	↑
Bouassida *et al.* (2003)	RUN	SS (21 min @ 70%, 21 min @ 85%)	42 min			
@ 0 h post-ex				↑	↔	↓
@ 24 h post-ex				↑	↔	↔
Guillemant *et al.* (2004)	CYC	SS (80% VO_{2max})	60 min	↑	↑	NM
Maïmoun *et al.* (2005)	WALK	INC to exhaustion	8-12 min	↑	NM	↓
Maïmoun *et al.* (2006)	CYC	SS (85% or 115% VT)	50 min			
@ 85% VT				↔	↔	NM
@115% VT				↑	↔	NM
Barry *and Kohrt* (2007)	CYC	60-75% VT	2 h			
without PV correction				↑	↑	NM
with PV correction				↑	↓	NM
Maïmoun *et al.* (2009)	WALK	INC to exhaustion	8-12 min	↑	NM	↓

PTH, parathyroid hormone, PTH; tCa, total serum calcium; iCa, ionised calcium; ↑, increase; ↓ decrease; ↔, unchanged; bpm, beats per min (heart rate); CYC, cycling; INC, incremental; INT, intermittent; NM, not measured; PV, plasma volume; RM, repetition maximum; RUN, running; RES, resistance exercise; SS steady-state; VO_{2max}, maximum oxygen uptake; WALK, walking; W_{max}, workload max; VT, ventilatory threshold.

Grimston *et al.* (1993) showed an increase in PTH despite no change in total calcium, but only in trained female runners with low BMD. In those with normal BMD, there was no change in either total calcium or PTH. Salvesen *et al.* (1994) report increased total calcium with increased PTH in response to a incremental, 50 min treadmill run. They also observed increased total calcium with no change in PTH in fire fighters, and increased total calcium and PTH in endurance-trained runners during the same, constant velocity running test. Kristofersson *et al.* (1995) report increased iCa after 30 sec of maximal effort cycling but observed no change in either total calcium or PTH. Compared to a resting control group, PTH is increased with an acute bout of resistance exercise despite no change in iCa, although PTH is unchanged despite an increase in iCa with 15 min cycling at 85% of VO_{2max}. Forty-five min of cycling at 55% VO_{2max} had no effect on either PTH or iCa (Rong *et al.*, 1997).

Thorsen *et al.* (1997) report no change in PTH despite decreased iCa at 1 h after exercise, while at 24 h post-exercise, iCa was no longer reduced but PTH was increased. At 72 h post-exercise, iCa was again decreased while PTH remained increased.

iCa is decreased and total calcium increased immediately after a bout of resistance exercise with no change in PTH (Ashizawa *et al.*, 1997). Subsequently, iCa was increased at 45 min post-exercise when PTH and total calcium were unchanged whilst at 105 min post-exercise, PTH was decreased but neither total calcium or iCa were different from pre-exercise values. Brahm *et al.* (1997a) report increased PTH despite increased total calcium immediately after a 35 min, incremental treadmill run. In the same year they report no change in either total calcium or PTH in response to a bout of one-legged knee-extension exercise (Brahm *et al.*, 1997b). The increases in PTH with a VO_{2max} test seen before and after 6 weeks of endurance training are both accompanied by an increase in total calcium (Zerath *et al.*, 1997). Tsai *et al.* (1997) report increases in both total calcium and PTH following 60 min of cycling exercise at 60% VO_{2max} while PTH is increased with no change in total calcium or iCa after moderate jogging, but unchanged despite increased total calcium and iCa with an incremental cycling test to exhaustion (Rudberg *et al.*, 2000).

iCa is decreased and total calcium unchanged with 21 min of exercise at 70% VO_{2max} that results in increased PTH (Bouassida *et al.*, 2003). PTH remains increased and iCa decreased after a further 21 min of exercise at 85% VO_{2max} although the magnitude of both is reduced when a 40 min break is included between the two bouts. With the break included, PTH remains elevated at 24 h post-exercise despite iCa returning to pre-exercise levels. Guillemant *et al.* (2004) observed a 2.5 to 3-fold increase in PTH with 60 min of cycling at 80%VO_{2max} but this increase was accompanied by no change in the total calcium. When subjects drank high-calcium mineral water before and throughout exercise iCa increased by 8% but PTH concentrations were still increased by ~70%.

A VO_{2max} test is associated with decreased iCa and increased PTH immediately after exercise (Maïmoun *et al.*, 2005; 2009). Maïmoun *et al.* (2006) report no effect of 50 min of cycling at 85% of VT on either total calcium or PTH, but PTH is increased and total calcium unchanged with exercise at 115% of VT. Finally, Barry and Kohrt, (2007) observed increases in both total calcium and PTH immediately after 2 h of cycling at 60-75% of VT, although following correcting for changes in plasma volume, PTH remained increased but total calcium concentrations were reduced from pre-exercise levels. As the calcium-sensing receptor in the parathyroid gland responds to the concentration rather than the total amount of iCa, it might not be appropriate to correct total calcium concentrations for changes in plasma volume. Even with this correction, however, there was no correlation between changes in the two variables.

2.6.5.2 Phosphate

Increased phosphate PO_4 concentrations can also result in increased PTH concentrations. Until recently, it was not known if this effect was direct or indirect, as PO_4 loading is also associated with hypocalcemia, skeletal resistance to PTH and the reduced suppression of calcitriol synthesis. In studies of PO_4 ingestion for example, Reiss *et al.* (1970) report that 1 h after the ingestion of 1000 mg of PO_4 that increased PO_4 levels by 14 to 40%, PTH is increased 60 to 125% and this increase is preceded or accompanied by decreased iCa and total calcium. Ittner *et al* (1986) reported a twofold increase of serum PTH and a decrease in serum calcium in normal adults whose serum PO_4 increased by 1.7 $mg \cdot dl^{-1}$ after ingesting 1500 mg of PO_4. Studies of PO_4 infusions report similar findings, with an increase in the systemic PO_4 concentration increasing PTH and decreasing Ca (Kaye, 1995).

In contrast, Silverberg *et al.*, (1986) report a significant increase in serum PO_4 without significant changes in total calcium and PTH in young adults, 1 h after a 1000 mg dose of PO_4, although 2 g of PO_4, on 5 consecutive days, increased serum PO_4 by 26% and PTH by 50%. More recently, Calvo and Hunter Heath III, (1988) report no change in either iCa or PTH in the 4.5 h following the ingestion of 1000 mg of PO_4 that increased serum PO_4 by 35 to 40%. When the dose was increased to 1500 mg, serum PO_4 only increased by ~25% but there was a significant although transient increase in PTH at 1 h. Ionised calcium was decreased by PO_4, although the nadir in iCa concentrations followed, rather than preceded, the peak in PTH.

Other studies appeared to indicate that the effect of PO_4 on PTH was indirect. For example, in the study of Reiss *et al.* (1970), the increase in PO_4 correlated poorly with the increase in PTH and, in a secondary experiment, oral PO_4 failed to increase serum PTH when hypocalcemia was prevented by a calcium infusion. Similarly, a PO_4 infusion concomitantly with calcium control resulted in no change in PTH (Sherwood *et al.*, 1968). However, it is possible that these findings are confounded by the large amount of calcium that was infused and its independent effect on PTH release. Additionally, due to the age of these experiments and the subsequent improvements in the sensitivity of PTH assays, results may differ if these studies were to be repeated.

Both the studies of Reiss *et al.* (1970) and Sherwood *et al.* (1968) were based on very small subject populations and subsequently, both *in vitro* and *in vivo* evidence indicates that PO_4 can directly increase PTH concentrations. *In vitro* studies show that increased PO_4 concentration in the medium increases PTH secretion from both intact parathyroid glands and parathyroid gland slices (Almaden *et al.*, 1996; Nielsen *et al.*, 1996). Using a clamp to maintain iCa concentrations, Estepa *et al.* (1999) showed that a PO_4 infusion increased PTH independently of iCa in dogs. This finding corroborated the findings of several previous studies that had shown PO_4 restriction corrected the secondary hyperparathyroidism associated with experimentally induced chronic kidney disease (CKD) in dogs

(Slatopolsky and Bricker, 1973; Lopez-Hilker *et al.*, 1990), and did so independently of changes in serum calcium and 1,25 $(OH)_2$ vitamin D (Lopez-Hilker *et al.*, 1990), and prevented the increase in serum PTH levels in CKD patients (Portale *et al.*, 1984; Lucas *et al.*, 1986).

In the study of Estepa *et al.* (1999), increases in circulating PO_4 of 270% and 370% were associated with 50% and 150% increases in PTH, whereas a 100% increase had no effect on PTH. In a more recent study in rats, however, an intravenous infusion of PO_4 resulted in a 3-4-fold increase in PTH despite an increase in PO_4 of only 25% (Martin *et al.*, 2005) suggesting that, in animals at least, PTH concentrations are can be stimulated by more subtle increases in circulating PO_4 levels.

In humans, Fine *et al.* (1993) observed a 65% increase in PTH when the PO_4 concentration of the dialysate of haemodialysis patients was increased by 40% over a 10 to 12 week period. Although only 50% of patients responded to PO_4 with an increase in PTH, those that did showed no change in serum iCa. Kärkkäinen and Lamberg-Allardt, (1996) reported on changes in PTH and iCa in response to 1500 mg of PO_4, given either in a single dose of three separate doses. Increased PTH concentrations were observed in both conditions, with increases in PO_4 of 15-36% and PTH increased in both conditions. Although iCa was decreased in the single dose condition, it was unchanged in three dose condition suggesting a direct effect of PO_4 on PTH.

There is considerable evidence of a physiological relationship between PO_4 and PTH in humans. A prolonged fast results in a loss of the rhythms of both PTH and serum PO_4 (Fraser *et al.*, 1994). In postmenopausal women with established osteoporosis, the acrophase for serum PO_4 precedes that for PTH suggesting a possible role for serum PO_4 in the generation of the circadian rhythm of PTH (Fraser *et al.*, 1998). Subsequently, using cross-correlational analysis, the strongest relationship between PTH and PO_4 in healthy subjects is observed when PTH is lagged by 1.5 h further suggesting a relationship between changes in PO_4 and subsequent changes in PTH (Ahmad *et al.*, 2003). It has also been suggested that the abnormalities – a blunted nocturnal increase in PTH concentration and a sustained afternoon/evening PTH increase – observed in the PTH circadian rhythm in treated and untreated adult growth hormone deficiency (AGHD) patients may be related to the blunted nocturnal increase and sustained afternoon/evening increase in PO_4 concentrations (White *et al.*, 2007).

Although the majority of studies of acute exercise report an increase in PO_4 concentrations with acute exercise, its association with increased PTH is less clear. A summary of these studies is provided in Table 2.10. Ljunghall *et al.* (1986) report increases in both PO_4 and PTH immediately after 5 h of exercise at 40 to 50% VO_{2max}. Likewise, Nishiyama *et al.* (1988) report increases in both PO_4 and PTH immediately after 30 min of running at 50% effort while Tsai *et al.* (1997) and Rudberg *et al.* (2000) observed increased PTH and PO_4 with moderate cycling and jogging respectively. The 11% increase in PTH immediately after a VO_{2max} is accompanied by a 39% increase in PO_4

(Zerath *et al.*, 1997). However, after 6 weeks of endurance training, the PTH response to the same test was increased to 22% despite a similar increase (44%) in PO_4 to that seen prior to training. Rudberg *et al.* (2000) also report no change in PTH despite a 30% increase in PO_4 in response to cycling exercise. Similarly, Kristofersson *et al.* (1995) report a ~20% increase in PO_4, 5 min after short duration sprint exercise but no change in PTH and even a tendency for decreased concentrations. Maïmoun *et al.* (2006) observed significant increases in PO_4 during 50 min of cycling at both 85% and 115% VT. However, although the magnitude of this increase was similar in both conditions, there was no increase in PTH at 85% VT but a progressively increase in PTH at 115%.

Table 2.10. Changes in parathyroid hormone (PTH) in relation to changes in phosphate (PO_4) with acute exercise.

Reference	Exercise Protocol			PTH	PO_4
	Type	*Protocol*	*Duration*		
Ljunghall *et al.* (1986)	CYC	SS (40-50% W_{max})	5 h	↑	↑
Nishiyama *et al.* (1988)	RUN	43-52% of maximum effort	30 min	↑	↑
Kristofersson *et al.* (1995)	CYC	Maximum effort	30 sec	↔	↑
Tsai *et al.* (1997)	CYC	SS (60% VO_{2max})	60 min	↑	↑
Zerath *et al.* (1997)	CYC	INC to exhaustion (VO_{2max} test)	NS		
Before 6 wk endurance training				↑	↑
After 6 wk endurance training				↑	↑
Ashizawa *et al.* (1997)	RES	3 sets of seven exercises (60-80%	45 min		
@ 0 h post-ex		of 1 RM)		↔	↓
@ 45 min post-ex				↔	↓
@ 105 min post-ex				↓	↓
Rudberg *et al.* (2000)	RUN	SS (96–148 bpm)	30-40 min	↑	↑
	CYC	INC to exhaustion	(20-32) min	↔	↑
Guillemant *et al.* (2004)	CYC	SS (80% VO_{2max})	60 min	↑	↑
Maïmoun *et al.* (2006)	CYC	SS (85% or 115% VT)	50 min		
@ 85% VT				↔	↑
@115% VT				↑	↑

PTH, parathyroid hormone; PO_4, phosphate; ↑, increase; ↓ decrease; ↔, unchanged; bpm, beats per min (heart rate); INC, incremental; CYC, cycling; RES, resistance exercise; RM, repetition maximum; RUN, running; SS steady-state; VO_{2max}, maximum oxygen uptake; VT, ventilatory threshold; W_{max}, workload max.

Guillemant *et al.* (2004) showed a 37% increase in PO_4 during 60 min of cycling at 80% VO_{2max} accompanying the 2.5-3 fold increase in PTH. However, although calcium ingestion before and during exercise had no effect on the increase in PO_4 (+42%), the increase in PTH was reduced to only 80%. The only study not to report an increase in PO_4 with exercise was that of Ashizawa *et al.* (1997)

who observed a decrease in PO_4 from 15 to 105 min after a bout of resistance exercise. There was also no increase in PTH with exercise, although concentrations were decreased at 105 min post-exercise.

In summary, although PO_4 is consistently increased with bouts of acute exercise, this increase is not always associated with increases in PTH although, based on data from PO_4 ingestion and infusion studies in animals and humans (Silverberg *et al.*, 1986; Kärkkäinen and Lamberg-Allardt, 1996; Martin *et al.*, 2005), it appears that the magnitude of the change in PO_4 with exercise (up to approximately 50%) might well be sufficient to induce an increase in PTH.

2.6.5.3 Glycaemic status

Insulin-induced hypoglycaemia can affect circulating PTH levels although there are conflicting results regarding the nature of this relationship. Studies in humans during acute hypoglycaemia report an increase (Shah *et al.*, 1975; Ljunghall *et al.*, 1984a), a decrease (Body *et al.*, 1983), or no change (Broulik *et al.*, 1987) in plasma PTH. This relationship may be complicated by the stimulating effect of hypoglycaemia on catecholamines (Cryer, 1980), which may have independent effects on PTH secretion (Fischer *et al.*, 1973). Poor assay specificity for PTH when using conventional RIAs might explain some of the discordance in results, as using a specific immunometric assay, Shearing *et al.* (1992) report a rapid and marked reduction in PTH during insulin-induced hypoglycaemia. This response was preserved during α-adrenoceptor and β-adrenoceptor blockade although its magnitude was reduced suggesting that hypoglycaemia suppresses PTH independently of catecholamines.

Changes in PTH during insulin-induced hypoglycaemia may be related to changes in circulating PO_4 (Nowicki *et al.*, 1998) resulting from insulin-stimulated glucose disposal, as when the decrease in PO_4 during hyperinsulinemia is prevented by a concomitant infusion of PO_4, PTH concentrations remain unchanged (Nowicki *et al.*, 1996). The suppressive effect of hypoglycaemia on PTH was confirmed by Clowes *et al.* (2002b) during a hypoglycaemic, hyperinsulinemia clamp and, in agreement with Nowicki *et al.* (1998), the authors also observed PTH to be suppressed during a euglycaemic, hyperinsulinemia clamp, although to a lesser extent than during hypoglycaemic, hyperinsulinemia. This suggests that the effect of hypoglycaemia on PTH is mediated by changes in PO_4 resulting from hyperinsulinemia and enhanced by the combination hyperinsulinemia and hypoglycaemia.

The role of glycaemic status in changes in PTH with acute exercise is unclear. Moderate exercise increases insulin concentrations which might be expected to suppress PTH concentrations, but the majority of studies report increased rather than decreased PTH concentrations with exercise (Table 2.6.4). Importantly, hypoglycaemia during or immediately after exercise is rare, occurring only with strenuous endurance cycling but not with strenuous running and typically glucose levels do not decrease as low an those in insulin-induced hypoglycaemia studies (Shearing *et al.*, 1992; Clowes *et al.*, 2002b). Additionally, when hypoglycaemia does incur with strenuous exercise, it is typically

accompanied by markedly reduced insulin concentrations. If changes in PTH during hypoglycemia are mediated by changes in PO_4, PTH concentrations might be expected to decrease during hypoglycaemic, hypoinsulinemia as hypoinsulinemia would reduce serum PO_4 levels through a decrease renal PO_4 reabsorption (Allon and Shanklin, 1992) and hypoglycaemia and would have the same effect through increased cellular uptake. That said, the later effect might be counteracted to some degree by the exercise-induced hypoinsulinemia, which might inhibit cellular PO_4 uptake.

2.6.5.4 Metabolic acidosis

Metabolic acidosis can also provoke increases in PTH. PTH administration has been shown to increase net acid excretion (Hultner, 1985; Stim *et al.*, 1994) and induce metabolic alkalosis in dogs and humans by both renal and extrarenal mechanisms (Hultner *et al.*, 1982; Hultner *et al.*, 1983; Hultner and Petersen, 1985). In rats, a 3 h infusion of hydrochloric acid that reduced arterial pH from 7.27 ± 0.02 to 7.19 ± 0.02 increased PTH concentrations by ~50% despite an increase in iCa (Bichara *et al.*, 1990). In dogs, a 60 min induction of metabolic acidosis during which blood pH declined, rapidly increased PTH values by approximately 3-fold (López *et al.*, 2002). In a subsequent study (López *et al.*, 2003), the induction of metabolic alkalosis that progressively increased blood pH, decreased rapidly PTH by ~75%. As metabolic acidosis is known to increase iCa concentrations due to the pH dependency of calcium binding, these studies clamped iCa at normal levels indicating that acute changes in blood pH rapidly affect PTH secretion independently of changes in calcium.

The physiological increase in the iCa concentration that occurs during the induction of metabolic acidosis reduces, but does not eliminate, the acidosis-induced increase in PTH secretion (López *et al.*, 2004). In this study, a decrease in blood pH of ~0.10 units to about 7.30 resulted in near-maximal PTH stimulation. There is comparatively less data on the PTH response to acute metabolic acidosis in humans. In one study, following the ingestion of 1.5-1.75 $mEq \cdot kg^{-1}$ body mass of ammonium chloride, given orally over 1 h, PTH was increased by ~20% although no resulting blood pH data was reported in this study (Coe *et al.*, 1975). From these and other data, it has been proposed that the increased PTH secretion during metabolic acidosis may increase bone resorption to provide additional buffer base in extracellular fluid and may increase renal PO_4 clearance to augment urinary buffering capacity and acid excretion (Wachman and Bernstein, 1970; Wills, 1970). However, although PTH may be elevated by metabolic acidosis, it potency to induce end organ effects might also be diminish, as evidenced by reduced phosphaturic response to PTH follow both ammonium chloride treatment (Beck *et al.*, 1975) and fasting (Beck *et al.*, 1979). The diminished response has been shown to be related to the inhibition of the PTH-dependent cyclic AMP system at the level of adenylate cyclase in the renal cortex (Beck *et al.*, 1975).

Increases in blood and muscle lactate, and the coincident decrease in pH with intense exercise, have traditionally been attributed to the production of lactic acid. More recently, an increased reliance on non-mitochondrial ATP turnover has been proposed to underlie the biochemistry of exercise-induced metabolic acidosis (Robergs *et al.*, 2004), although this interpretation has subsequently been questioned (Kemp, 2005). The effect of intense exercise on blood pH has been studied in detail and studies show a fall in pH during exercise. For example, short (< 2 min), high intensity exercise results in a significant drop in blood pH with post-exercise pH values of between 7.04 and 7.18 units (Hermansen and Osnes, 1972; Sahlin *et al.*, 1976; King *et al.*, 1985; Costill *et al.*, 1983; Nevill *et al.*, 1989; Allsop *et al.*, 1990). Exercise of this nature is accompanied by blood lactate concentrations in excess of 15 $mmol \cdot L^{-1}$.

There is considerably less information regarding changes in blood pH during longer-duration, endurance-type exercise where steady-state blood lactate levels achieved that are considerably lower than those in the studies described above. For example, after 10 min of running at maximum lactate steady state (MLSS, ~80% VO_{2max}) pH levels are 7.31 increasing to 7.35 by 30 min with lactate concentration of 4 to 5 $mmol \cdot L^{-1}$ throughout (Peinado *et al.*, 2006). Similar pH values and pattern of change are reported during 30 min of cycling at MLSS (Baron *et al.*, 2003).

Several studies have simultaneously examined changes in blood pH and PTH concentrations, a summary of which is provided in Table 2.11. Cunningham *et al.* (1985) reported a decrease in blood pH from 7.42 to 7.18 at 1 min after 1to 2 min of exhaustive treadmill running but no change in PTH. Like in unclamped, resting conditions, however, acidosis in this study was accompanied by an increase in the iCa concentration that might have mediated the PTH response to acidosis. Following a 20 min incremental run up to 90% VO_{2max}, Henderson *et al.* (1989) report a blood pH value of 7.27 accompanied by an 85% increase in PTH despite an increase in iCa.

Rong *et al.* (1997) compared pH and PTH responses to 45 min cycling at 55% VO_{2max}, 15 min cycling at 85% VO_{2max} and leg resistance exercise at 85% of three-repetition max (RM). Cycling at 55% VO_{2max} had no effect on either blood pH or PTH concentrations while cycling at 85% VO_{2ma} reduced pH to ~7.32 but has no effect on PTH. Resistance exercise reduced blood pH to 7.35 and produced a transient increase in PTH. Rudberg *et al.* (2000) report a significant decrease in blood pH from 7.37 to 7.32 immediately after a brief bout of incremental cycling to volitional exhaustion but observed no change in PTH concentrations. In contrast, PTH was increased by ~50% despite no change in blood pH following 30 to 40 min of moderate jogging. This difference might, in part, be explained by the increase in iCa seen with cycling but not jogging. Guillemant *et al.* (2004) report significant increases in PTH with 60 min of cycling at 80% VO_{2max} but observed no significant change in blood pH (minimum values of 7.38 to 7.36).

Table 2.11 Changes in parathyroid hormone (PTH) in relation to blood pH with acute exercise.

Reference	Exercise Protocol			PTH	pH*
	Type	*Protocol*	*Duration*		
Cunningham *et al.* (1985)	RUN	Maximum effort until exhaustion	60-130 sec	↔	7.18
Henderson *et al.* (1989)	CYC	INT, INC (40-90% VO_{2max})	20 min	↑	7.27
Rong *et al.* (1997)	RES	5 sets of eight reps (85% of 3 RM)		↑	7.35
	CYC	SS (85% VO_{2max})	15 min	↔	7.32
	CYC	SS (55% VO_{2max})	45 min	↔	↔
Rudberg *et al.* (2000)	RUN	SS (96–148 bpm)	30-40 min	↑	↔
	CYC	INC to exhaustion	(20-32) min	↔	7.32
Guillemant *et al.* (2004)	CYC	SS (80% VO_{2max})	60 min	↑	↔

PTH, parathyroid hormone; pH, blood pH; * lowest value reported (significantly reduced from pre-exercise); ↑, increase; ↓ decrease; ↔, unchanged; bpm, beats per min (heart rate); CYC, cycling; INC, incremental; INT, intermittent; RES, resistance exercise; RM, repetition maximum; RUN, running; SS steady-state; VO_{2max}, maximum oxygen uptake; VT, ventilatory threshold; W_{max}, workload max.

2.6.5.5 Adrenergic agonists

The role of the adrenergic system in changes in systemic PTH remains unclear. Although a number of studies have indicated that PTH secretion can be modulated by the adrenergic system, the evidence for the direction of this effect is inconsistent. Using epinephrine and norepinephrine, as well as adrenergic agonists and antagonists such as isoproterenol (β agonist) and propranolol (β blockade), these studies have shown that adrenergic agonists increase PTH whilst blocking agents suppress basal PTH and attenuate the PTH response to agonists (Fischer *et al.*, 1973; Kukjera *et al.*, 1975; Blum *et al.*, 1978; Metz *et al.*, 1978). Other studies, however, report either no effect on PTH (Epistein *et al.*, 1983) or the suppression of PTH secretion (Christensen *et al.*, 1977).

Non-pharmacologic studies indicate that the sympathetic nervous system predominantly exerts an attenuating effect on PTH. For example, chronic sympathetic denervation increases plasma PTH in rats (Stern *et al.*, 1993) and in humans, plasma PTH levels are suppressed by activation of the sympathetic nervous system using lower body negative pressure (Joborn *et al.*, 1987). More recently, Schmitt *et al.* (2003) report that acute β-receptor blockade increases plasma PTH by augmenting PTH burst mass. Blockade has no effect on pulse frequency or orderliness suggesting no involvement of the sympathetic nervous system in the generation of PTH pulses *per se*.

The infusion of epinephrine results in dose-dependent increase in PTH (Fischer *et al.*, 1973). Epinephrine, however, is a non-selective agonist of all adrenergic receptors and the use of selective alpha (methoxamine and phenylephrine) and beta (isoproterenol) agonists has shown that the effect on PTH is mediated through the β-adrenergic pathway with α-selective agonists having no effect (Kukjera *et al.*, 1975; Metz *et al.*, 1978). Higher doses of epinephrine result is a small reduction in serum Ca suggesting the effect of adrenergic stimulation on PTH might be indirect. However, when Ca concentrations were lowered with ethylene glycol tetraacetic acid (EGTA), a more pronounced decrease in Ca was required to produce an increase in PTH comparable to that with epinephrine (Fischer *et al.*, 1973).

As magnesium concentrations remained unchanged in these studies, this suggests that epinephrine has a direct effect on the parathyroid glands and is consistent with the presence of a rich adrenergic nerve supply to the parathyroid glands (Altenähr, 1971; Yeghiayan *et al.*, 1972,). Together with the data of Fischer *et al.* (1973), Blum *et al.* (1978) showed that although variations in calcium concentrations and β-adrenergic agonists modify PTH levels by two different and independent mechanisms, the two mechanisms modulated the effects of each other. Thus, while the magnitude of the acute PTH response to epinephrine was enhanced by mild hypocalcemia but suppressed by hypercalcaemia and severe hypocalcemia, infusions of epinephrine or isoproterenol enhanced the acute PTH response to EGTA-induced hypocalcemia when β-adrenergic agonists alone were no longer effective (Fischer *et al.*, 1973; Blum *et al.*, 1978). Norepinephrine also increases PTH but is less potent than either epinephrine or isoproterenol (Fischer *et al.*, 1973).

It is well established that an acute bout of exercise increases the circulating catecholamine concentrations. Catecholamine concentrations increase progressively during submaximal exercise, with peak concentrations occurring immediately post-exercise (Galbo *et al.*, 1975). Catecholamines increase at relatively low exercise intensities (40% VO_{2max}) and peak concentrations are increased with increasing exercise intensity (McMurray *et al.*, 1987). Similarly, during short-term incremental exercise, catecholamines increase exponentially with increasing exercise intensity (Galbo *et al.*, 1975; Mazzeo and Marshall, 1989) and a threshold for catecholamine accumulation has been described at intensity similar to that of the lactate and ventilatory thresholds (Mazzeo and Marshall, 1989; Weltman *et al.*, 1994). Improved training status is also known to enhance the catecholamine response to acute exercise (Zouhal *et al.*, 2008).

Body *et al.* (1983) observed that none of the studies described above report plasma epinephrine concentrations comparable to those achieved under physiologic conditions. In response, Body *et al.* (1983) administered epinephrine until steady-state concentration were achieved that spanned the physiological range (52 to 945 $pg \cdot mL^{-1}$) and saw no increase in PTH. This suggests that epinephrine does not play a physiologic role in the regulation of PTH although these experiments have not subsequently been repeated using the more sensitive PTH assays currently available.

Several studies of acute exercise appear to support the conclusion of Body *et al.* (1983). Vora *et al.* (1983) observed increases in catecholamines following maximal treadmill exercise but observed no change in PTH either during or after exercise. Ljunghall *et al.* (1984b) didn't measure catecholamines but observed no changes in PTH during maximal, incremental exercise with and without β blockade by propranolol. Subsequently, Joborn *et al.* (1988) observed a small and transient increase in PTH that occurred at 5 min after a 2 min bout of maximal effort one-legged knee extensions, was unaffected by β blockade with propranolol or verapamil.

Although these studies suggest no physiologic role for the adrenergic system on the PTH response to exercise, this interpretation might be complicated by the hypercalcaemia that occurred during exercise in all three studies because, as described above, the secretory response of PTH to β-adrenergic stimulation is suppressed when the extracellular calcium concentrations are raised *in vivo* (Fischer *et al.*, 1973).

2.6.6 The effect of acute exercise on circulating OPG

Cell system studies have shown that OPG expression at both the gene and the protein level is regulated by mechanical stimulation (Kim *et al.*, 2006; Saunders *et al.*, 2006), possibly via a mechanism involving mechanically-challenged osteocytes (You *et al.*, 2008). To date, however, only four studies have investigated the effect of acute exercise on OPG concentrations (Ziegler *et al.*, 2005; Brooke-Wavell *et al.*, 2007; Kerschan-Schindl *et al.*, 2009; Philippou *et al.*, 2009). These studies all measured sRANKL as well but, in contrast to clinical studies that report sRANKL is undetectable in 40-70% of samples, none of these studies report problems detecting sRANKL from either pre- or post-exercise blood samples.

Serum OPG concentrations are reported to be unchanged in non-elite runners immediately after a 15 km run, but increased by ~85% in a similar group of runners who completed the marathon distance (42.195 km). Over the shorter distance, there was no change in RANKL concentrations whereas at the end of the marathon, there was a 35% reduction in RANKL. Brooke-Wavell *et al.* (2007) report a much smaller (~10%) increase in OPG, 30 min after a bout of resistance exercise, with concentrations returning to pre-exercise levels at 24 h post-exercise. No changes in RANKL were observed in response to exercise. Philippou *et al.* (2009) observed an increase in OPG at 6 h following a single bout of muscle-damaging exercise. OPG returned to pre-exercise levels on 1 day after exercise, were elevated again at 2 days but not at 5 days post-exercise. RANKL showed a reciprocal pattern of change with concentrations decreased rather than increased. This resulted in an increase in the OPG to RANKL ratio at 6 h post-exercise only.

In the recent study of Kerschan-Schindl *et al.* (2009), OPG concentrations had increased ~150% immediately after a 246 km running race and remained ~100% higher that pre-exercise levels the following morning. In contrast, RANKL concentrations were unchanged immediately after the race

but were ~50% higher than pre-race levels the morning after. This study is the only investigation to date to measure changes in OPG and RANKL in concert with BTM in response to acute exercise. Despite the methodological limitations of this study described previously, regarding the standardisation of the nutritional status of subjects before blood samples and the timing of samples themselves, and, these findings suggest that that the sustained increase in bone resorption is accompanied by an increase in OPG immediately after the race and then by an increase in both OPG and RANKL at three days after the start of the race.

2.7 Summary

This review has shown that a single bout of acute exercise is associated with alterations in a variety of BTM including recently identified specific markers of both bone resorption and formation. However, despite the potent effects, both positive and negative, of exercise on bone health, whilst the volume of literature in this field continues to expand, it remains relatively understudied compared to other areas of bone biology. This relative paucity of research has resulted in few studies of comparable design in terms of the subjects studied, the exercise protocol used and the timings of the measures of BTM. Interpretation of these studies is further confounded by the continuing improvement in the specificity of BTM that has led to only a small number of studies that have used specific markers such as β-CTX and P1NP (Table 2.12).

Table 2.12. Summary of key findings from literature review

Literature area	Summary
Effects of acute exercise on specific bone turnover markers	Few studies have measured specific bone resorption (*e.g.* β-CTX, DPD) and formation (*e.g.* P1NP, bone ALP) markers in response to acute exercise, with information on bone formation markers being particularly sparse. Plasma β-CTX increases with endurance cycling but the time course of this increase remains unclear while the response of these markers to endurance running remains largely unknown. In existing studies, the degree of control for factors known to affect the pre-analytical variability of bone markers particularly plasma β-CTX (*e.g.* acute nutritional status, circadian rhythm), varies considerably.
Effects of acute exercise on circulating OPG concentrations	Increased circulating OPG concentrations might reflect increased bone resorption. Circulating OPG concentrations might increase during endurance running but the time course of this response, and its relationship with changes in bone turnover markers under controlled conditions, remains unknown.
Effects of acute exercise on circulating PTH concentrations	Acute exercise increases PTH concentrations and there is evidence that this increase might be related to exercise intensity. The time course of this increase, particularly in the hours that follow exercise, remains to be fully determined.

ALP, alkaline phosphatase; β-CTX, c-terminal telopeptide of type 1 collagen; DPD, deoxypyridinoline; OPG, osteoprotegerin; PTH, parathyroid hormone; P1NP, n-terminal propeptides of type 1 procollagen.

This, in turn, has led to a much smaller group of studies that have examined changes in the same, specific markers and compounds the issue of the wide variation in other aspects of study design. In addition, the measurement of these markers, particularly β-CTX, has introduced new sources of variability that some previous studies have not fully controlled for and may further affect the comparison of results. Finally, unlike earlier studies that used less-specific markers, more recent studies have not examined changes in specific BTM for periods in excess of 24 h.

OPG is known to increase during endurance running but the timecourse of this response, and its association with changes in BTM under controlled conditions remains unknown (Table 2.12). Therefore, the measurement of OPG concomitantly with specific markers of bone turnover under controlled conditions might provide supplementary information for the evaluation of OPG as an indirect marker of changes in bone turnover with running. The measurement of specific BTM in conjunction with PTH and its candidate systemic regulators might provide insights into the mechanisms by which exercise alters bone turnover and therefore bone structure. It is clear, therefore, that further investigations which measure specific BTM under conditions that attempt to control for factors that contribute significantly to the pre-analytical variability in their measurement, will provide novel information concerning the bone metabolic response of bone to running.

CHAPTER III

GENERAL METHODOLOGY

3.1 General Study Design

3.1.1 Introduction

The four studies used an identical general research design that included two preliminary visits and between one and three experimental conditions consisting of eight or nine consecutive days. During the first preliminary visit, subjects provided written informed consent, underwent a full medical examination (Section 3.2.1) and were provided with a food diary (Section 3.2.2). During the second preliminary visit, subjects were familiarised with study procedures and had their cardiorespiratory responses to level (0° gradient) treadmill running (Section 3.2.3) and maximal rate of oxygen uptake (VO_{2max}) (Section 3.2.4) determined. Volunteers then completed an experimental trial over eight consecutive days in Studies I, III and IV and nine days in Study II (Section 3.1.2). All studies were approved by the QinetiQ Research Ethics Committee.

3.1.2 Experimental trial overview

For the first three days of the experimental period (D1 to D3), subjects were required to refrain from all physical exercise and training and followed an individualised experimental diet (Section 3.2.5). On the fourth day (D4) (Study I, III and IV, Figure 3.1), and on D4 and D5 (Study IV, Figure 3.2), subjects completed an exercise intervention on a treadmill. A second-void urine sample was collected prior to exercise and blood samples were collected before, during and after the intervention (Sections 3.3.2 and 3.3.3).

On the final four days (D5 to D8 in Study I, III and IV and D6 to D9 in Study II) subjects continued to refrain from exercise and training, again followed their individualised diet, and made a morning visit to the laboratory where a blood and second-void urine sample were collected.

Blood samples were analysed for β-CTX, P1NP, bone ALP, OC, OPG, intact PTH, albumin-corrected calcium (ACa), PO_4, cortisol, leptin, ghrelin, glucose and lactate (Section 3.3.4). Urine samples were analysed for fPYD, fDPD and creatinine (Cr).

Subjects agreed not to take any vitamin or mineral supplements from seven days before their first condition until completion of the study. Subjects were asked to report to the investigator any symptoms of illness or fever in the days leading up to each experimental condition, and if such an episode occurred, the trial was postponed until subjects were clear of symptoms for a minimum of five days.

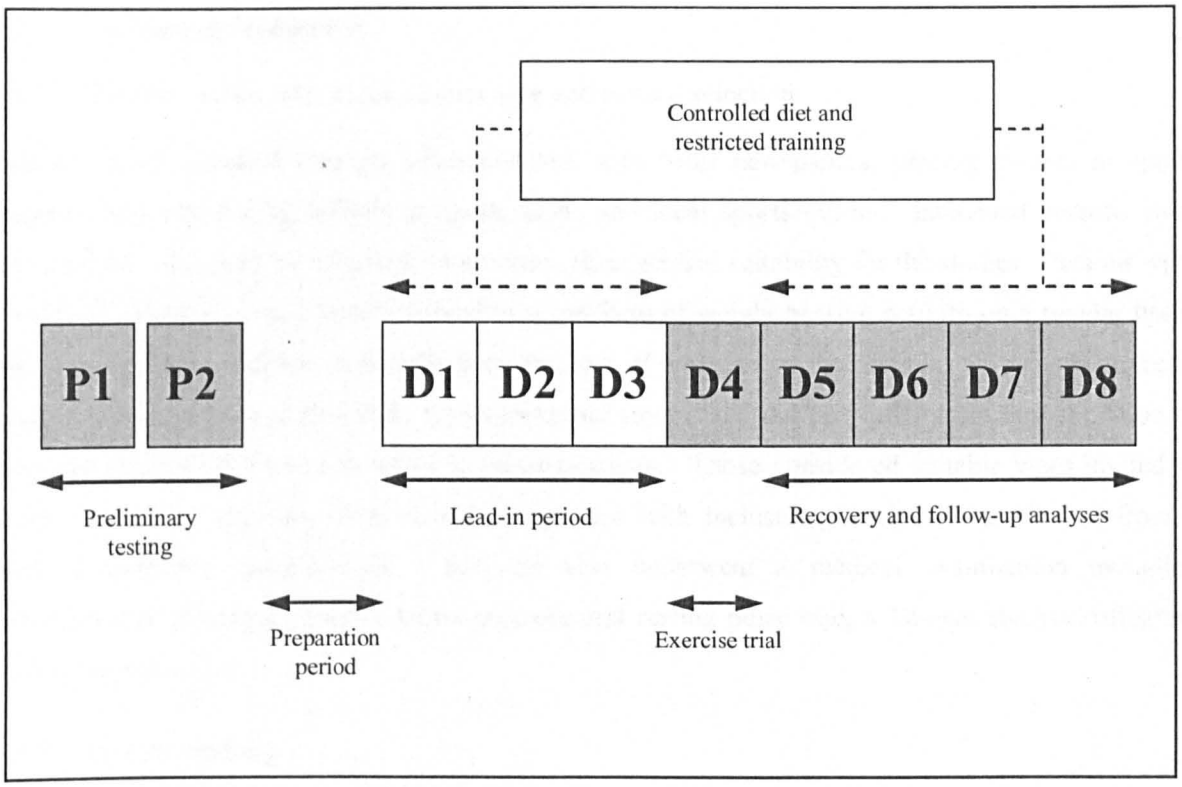

Figure 3.1 Outline of general study design in Study I, III and IV. P1 to P2, preliminary days; D1 to D8, experimental days. Shaded boxes denote laboratory visits; adjoining boxes denote consecutive days.

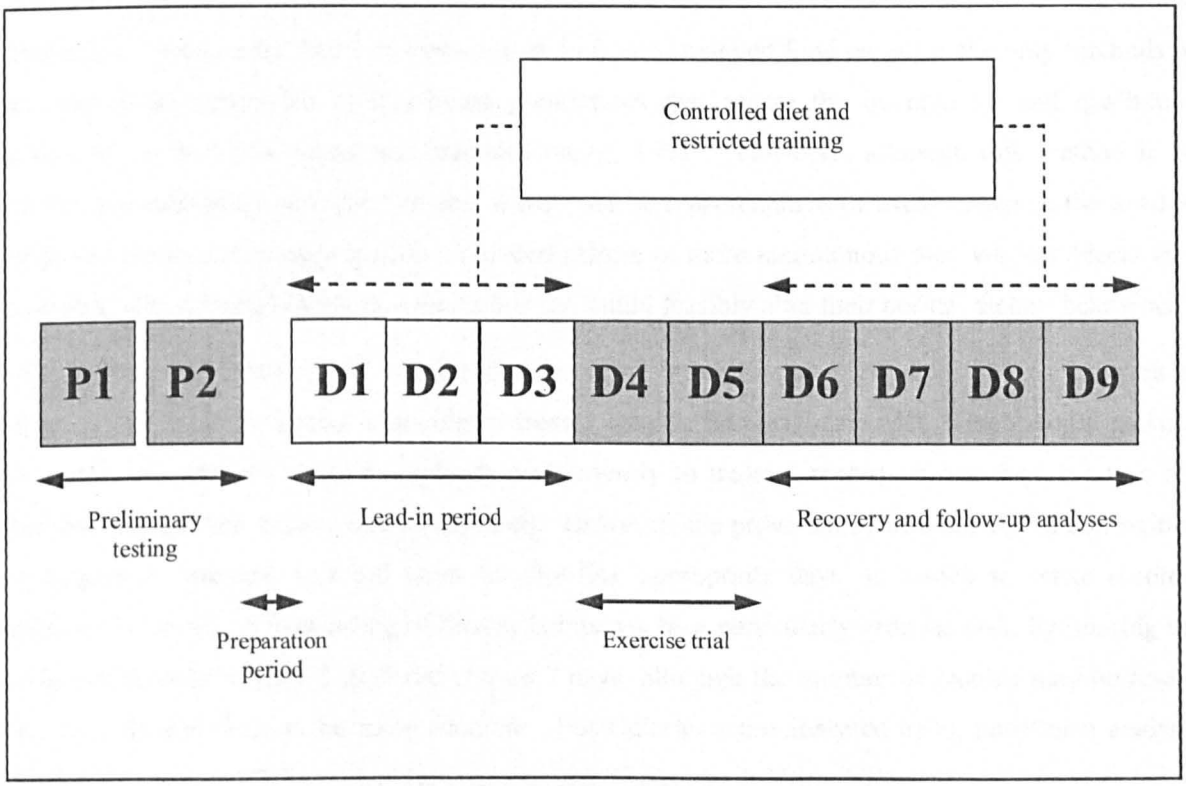

Figure 3.2 Outline of general study design in Study II. P1 and P2, preliminary days; D1 to D9, experimental days. Shaded boxes denote laboratory visits; adjoining boxes denote consecutive days.

3.2 Preliminary measures

3.2.1 Subject recruitment, medical screening and subject selection

Subjects were recruited through advertisements with local newspapers, placing posters in sports facilities and distributing leaflets at sports clubs and local sports events. Interested persons were subsequently contacted by telephone to ascertain their general suitability for the studies. Persons were considered suitable if they were involved in some form of weight-bearing activity on a regular basis, were non-smokers, had not suffered a bone fracture of any type in the previous 12 months, were in good physical health and free from musculoskeletal injury, and did not suffer from any condition or take any medication known to affect bone metabolism. Those considered suitable were invited to attend a medical screening session and compliance with inclusion criteria was confirmed from a medical screening questionnaire. Subjects also underwent a medical examination including measurements of height, weight, blood pressure and resting pulse rate, a 12-lead electrocardiogram (ECG) and urinalysis.

3.2.2 Dietary analysis

Subjects completed a three-day food diary consisting of two weekdays and one weekend day to calculate habitual daily energy intake (kcal) and macronutrient composition. Subjects were issued with a set of calibrated weighing scales to measure food intake, and received both verbal and written instructions. Along with direct observation, an in-home, weighed food record is the only methods of diet assessment applicable to free-living populations that assure the quantitative and qualitative validity of all nutrients consumed (Barrett-Connor, 1991). However, although this method is an accurate representation of current intake, it may not be representative of usual intake as the need to weigh and record intake may lead to a reduced calorie or more monotonous diet, while subjects who know their diet is being observed, even indirectly, might feasibly alter their normal dietary behaviour.

A further possible limitation of a 3 day dietary record rather that a longer recording period such as 7 days, is that average intake is calculated from a small a number of records, which would make it more prone to 'outliers' (*i.e.* if a subject inadvertently to make a record on one day that was not representative of their typical day-to-day diet). However, the provision of detailed verbal and written instructions to subjects will aid them in choosing appropriate days on which to make records. Additionally, as the self-recording of dietary habits can be a particularly arduous task, by limiting the number of records to only 3 days rather than 7 days, although the number of records may be fewer, these records are likely to be more accurate. Food diaries were analysed using nutritional analysis software (Microdiet V2; Downlee Systems Limited, Chapel-en-le-Frith, UK).

3.2.3 Determination of the association between oxygen uptake and running velocity during level (0° gradient) treadmill running.

To determine the association between oxygen uptake (VO_2) and running velocity during level running, subjects completed a 20 min continuous run on a treadmill (XELG 70 ERGO; Woodway, Waukesha, USA) consisting of four, 5 min stages. Sixty second samples of expired air were collected in the final minute of each stage using the Douglas bag technique. Heart rate was monitored continuously throughout the test using short range telemetry (Vantage NV, Polar Electro Oy, Kempele, Finland).

3.2.4 Determination of maximal rate of oxygen uptake

Maximal rate of oxygen uptake was determined using a discontinuous, incremental exercise test to exhaustion using a modified Taylor protocol (Froelicher *et al.*, 1974). Subjects completed a series of 3 min runs separated by 5 min rest periods. The treadmill gradient was increased by 2.5 % with each subsequent stage until volitional exhaustion was reached. Sixty second samples of expired air were collected in the final minute of each stage (1 min 45 sec to 2 min 45 sec) and during the final 30 sec of the last stage. Heart rate was monitored continuously throughout the test. In addition to volitional exhaustion, the criteria for attaining VO_{2max} were an increase in VO_2 of less than 0.15 $L \cdot min^{-1}$ with an increase in exercise intensity, a respiratory exchange ratio (RER) of >1.05, a heart rate of 220 – age (± 10 $beats \cdot min^{-1}$) and a blood lactate concentration at 1-2 min post-exercise of > 9 $mmol \cdot L^{-1}$. Using the results of the two tests, required treadmill velocities were estimated based on the regression line of VO_2 and treadmill velocity.

3.2.5 Experimental dietary provision

A diet, isocaloric with their habitual diet according to analysis of their food diary, was designed for each subject based on their individual dietary habits. Subjects were provided with three menus that were given in a 3 day cyclic order (individual macronutrient intakes are shown in Appendix A). During the experimental period, subjects provided their own food and were given both verbal and written instructions concerning the quantity of food and the preparation and timings of their meals. Subjects received a set of calibrated weighing scales to aid them with food preparation. Deviations from prescribed diets were recorded in a daily diary and confirmed verbally during visits to the laboratory from Day 4 onwards.

3.3 Trial Procedures

3.3.1 Metabolic measurements

Samples of expired air were collected into evacuated Douglas bags. The bags were emptied through a flow controller and volume counter and analysed for fractions of oxygen and carbon dioxide (Servomex 1400, Sussex, UK). The gas analyser was calibrated using certified reference gases (100 % Nitrogen; 16% O_2, 5% CO_2; BOC Gases, Surrey, UK). To allow the conversion of gas volumes from ambient temperature and pressure saturated (ATPS) to body temperature and pressure saturated (BTPS), measures of air temperature and pressure were also made.

3.3.2 Collection of blood and urine samples

On the day of the exercise intervention, blood samples were collected from an in-dwelling cannula (18GA 1.2x45 mm, Becton Dickinson, USA). The cannula was secured with tape, connected to a three-way tap (Connecta, Becton Dickinson, USA) and kept patent with an isotonic saline solution (0.9% NaCl). Prior to each blood sample being collected, 2 ml of blood was obtained and immediately discarded. Following each sample, the cannula was again flushed with saline. On the four recovery days (D5 to D8 in Study I, III and IV and D6 to D9 in Study II) blood samples were collected by venepuncture from a vein in, or just below, the anticubital fossa. Pre-exercise blood samples and those collected on the four recovery days were collected following an overnight fast from 2100 h the previous day. Urine samples were collected into sterile plastic containers.

3.3.3 Treatment and storage of blood and urine samples

For measurement of P1NP, β-CTX, OPG and cortisol, blood was transferred into pre-cooled tubes containing 15%, 0.12 ml of K_3E EDTA (Becton Dickinson Vacutainer System, USA) generating plasma. Tubes were gently inverted 8 to 10 times and centrifuged immediately at 2000 rpm and 5°C for 10 min. Plasma was separated and stored at -70°C until analysis.

For measurement of bone ALP, PTH, ACa, PO_4, leptin and ghrelin, blood was transferred into pre-cooled standard tubes (Becton Dickinson Vacutainer System, USA) and left to clot at room temperature for 60 min generating serum. Tubes were subsequently centrifuged at 2000 rpm and 5°C for 10 min and serum was separated and stored at -70°C until analysis.

For measurement of glucose and lactate, whole blood was transferred into pre-cooled tubes containing fluoride-oxalate generating plasma. Tubes were gently inverted 8 to 10 times and analysed in duplicate immediately (Yellow Springs Instruments, 2300 Stat Plus; YSI Inc., Yellow Springs, USA).

For measurement of fPYD, fDPD and Cr, a 20 ml sample of urine was decanted into a sterile plastic container and stored at -20 °C until analysis.

3.3.4 Biochemical Analysis

Biochemical analyses were conducted as described in Table 3.1

Table 3.1 Details of biochemical analysis methods

Measure	Method
β-CTX	Plasma β-CTX was measured using an ECLIA on an Elecsys 2010 immunoanalyser (Roche, Lewes, UK). Inter-assay coefficient of variation (CV) was <8% between 0.2 and 1.5 ug·L^{-1}. The assay sensitivity (replicates of the zero standard) was 0.01 ug·L^{-1}.
P1NP	Plasma P1NP was measured by an RIA supplied by Orion Diagnostica (Espoo, Finland). This assay has a sensitivity of 4 ug·L^{-1} established from precision profiles (22% coefficient of variation of duplicates) and an inter-assay CV of 3.5 to 5.4% across the concentration range 10 to 250 ug·L^{-1}.
OC	Plasma N-MID OC was measured using an ECLIA on a Modular Analytics E170 analyser (Roche Diagnostics, Lewes, UK). Inter assay CV is less than 5% between 2 and 200 ug·L^{-1}. The assay sensitivity (replicates of the zero standard) was 0.6 ug·L^{-1}.
Bone ALP	Serum Bone ALP was measured using a commercial immunometric assay (Metra Biosystems, Oxford, UK) with a sensitivity of 0.7 U·L^{-1} and a CV of less than 8% across the range 12 to 100 U·L^{-1}.
fPYD and fDPD	Urinary fPYD and fDPD were measured by a modification of the HPLC method described by Black *et al.* (1988). Acidified urine is applied to microgranular cellulose (CC31) in butanol (1/4) and washed before elution with heptafluorobutyric acid (0.1%). The eluent is then analysed by ion-pair reverse-phase HPLC using fluorescence detection. Acetylated PYD (Quidel/Metra Biosystems, Oxford, UK) is used as an internal standard. The interassay CV for both crosslinks methods was less 5.5% across the working concentration range for the assay. In all studies, pyridinoline results (in nmol·L^{-1}) are expressed relative to creatinine concentrations (fPYD/Cr and fDPD/Cr) as nmol·mmol Cr^{-1}. In Studies II-IV, pyridinoline 'output' (in nmol) was also calculated by multiplying pyridinoline concentrations (nmol·L^{-1}) by urine volume (L).
Cr	Creatinine was measured using a standard commercial assay (Roche, Lewes, UK) performed on a Roche Modular Analytical System. The range of measurement in urine is 360-57,500 umol·L^{-1}.
OPG	Plasma OPG was measured using a commercial solid phase ELISA supplied by IDS Boldon Tyne & Wear UK. The assay has a detection limit of 0.14 pmol·L^{-1} and an inter/intra assay CV of less than 10% across the range 1 to 30 pmol·L^{-1}.

Measure	Method
PTH	Serum PTH was measured using a commercial immunometric assay (Nichols Institute, San Juan, Capistrano, CA) with a detection limit of 0.5 $pmol \cdot L^{-1}$ and an inter-assay and intra-assay CV of less than 5% across the range 1 to 40 $pmol \cdot L^{-1}$.
Ca, albumin and PO_4	Serum Ca (range of measurement in serum of 0.05 to 5.00 $mmol \cdot L^{-1}$), albumin (range of measurement in serum of 10 to 70 $g \cdot L^{-1}$) and PO_4 (range of measurement in serum of 0.10 to 6.46 $mmol \cdot L^{-1}$) were measured using standard commercial assays (Roche, Lewes, UK) performed on a Roche Modular Analytical System.
Leptin	Serum leptin was measured in plasma using an EIA supplied by Immuno Diagnostic Systems (IDS) (Boldon UK). The assay has a sensitivity of 16 $ng \cdot mL^{-1}$ established from precision profiles (22% coefficient of variation of duplicates) and a CV of less than 8% across the range 30 to 500 $ng \cdot mL^{-1}$.
Ghrelin	Serum total (acylated and deacylated) ghrelin was determined in duplicate by a commercially available RIA (Phoenix Pharmaceuticals, Belmont, CA). Intra-assay and inter-assay CV were less than 10% across the working range of the assay.
Cortisol	Plasma cortisol was measured in plasma using ECLIA on a Roche Modular E170. The assay has a sensitivity of 8 $nmol \cdot L^{-1}$ established from precision profiles (22% CV of duplicates) and a CV of less than 6% across the range 16 to 1750 $nmol \cdot L^{-1}$.
Insulin	Insulin was measured in plasma by an electrochemiluminescence immunoassay (Roche, Lewes, UK) on a Roche Modular Analytical System. The assay has an intra-assay CV of 1.5-2.0% across the range 6.36 to 88.3 $mU \cdot L^{-1}$ and a total CV of 2.1-2.8% across the same range.

ECLIA, enzyme-linked chemiluminescent immunosorbent; EIA, enzyme immunometric assay; ELISA, enzyme-linked immunosorbent assay; HPLC, high performance liquid chromatography; RIA, radioimmunoassay.

Assay CVs were established from precision profiles (Ekins *et al.*, 1972; Ekins, 1977; Ekins, 1983). This method uses a response-error relationship to calculate the standard deviation in response as a function of the response. This standard deviation is divided by the slope of the dose–response calibration curve to generate a standard deviation in concentration units. Dividing the standard deviation in concentration units by the concentration at each point over the calibration range provides the typical representation of the precision profile as relative standard deviation (RSD) or CV *vs* concentration.

3.3.5 Statistical Analysis

The level chosen to indicate statistical significance was set at $P < 0.05$ and all data are presented as mean ± 1SD unless otherwise stated.

Analysis of subject characteristics, baseline biochemistry and variables relating to exercise varied depending on study design, and are described individually within each study chapter.

Body mass and biochemical data were analysed using a linear mixed model analysis of variance (LMM), with the factors *Time* (of sampling) and *Group* with individuals as a random, within-group effect. Prior to submission to the LMM, data sets were tested for assumptions of the LMM (homogeneity of variance and normality). If assumptions were not met, data sets were transformed until assumptions were satisfied.

Where the LMM indicated a significant effect of *Time* but no significant *Group* x *Time* interaction, data from all experimental groups was pooled and baseline values were compared to values at all subsequent time points using Dunnett's Test with baseline values as the '*Control*' group (Dunnett, 1964). When the LMM indicated a significant *Group* x *Time* interaction, baseline values within each individual group were compared to values at all subsequent time points using Dunnett's Test with baseline values as the '*Control*' group. Individual groups were then compared at each time point using the Student-Newman-Keuls (SNK) test. Pearson's correlation coefficient was calculated to examine the relationship between the percentage changes in PO_4 and PTH during exercise.

For the purposes of clarity, only significant differences between groups/conditions identified by the SNK test are displayed on figures. Significant differences from baseline in pooled data – or in individual groups if the LMM indicated a significant *Group* x *Time* interaction – identified by the Dunnett's test are summarised in the legend of each figure.

CHAPTER IV

STUDY I – THE EFFECT OF TRAINING STATUS ON THE METABOLIC RESPONSE OF BONE TO AN ACUTE BOUT OF EXHAUSTIVE RUNNING

4.1 Introduction

Given the generalised beneficial effects of exercise on bone health but also the reduced spinal BMD reported in some endurance-trained athletes (Bilanin *et al.*, 1989; Hind *et al.*, 2006), it is reasonable to expect endurance training to result in alterations in bone turnover. Cross-sectional studies report bone turnover to be higher in long distance runners compared to controls (Hetland *et al.*, 1993) and in active compared to inactive individuals (Nishiyama *et al.*, 1988), although a lower rate of bone turnover in endurance-trained runners compared to age-matched controls has also been reported (Brahm *et al.*, 1997c). Increased physical fitness is also associated with reduced basal PTH concentrations (Brahm *et al.*, 1997a) and enhanced exercise-associated increases in PTH (Zerath *et al.*, 1997) and bone formation markers (Brahm *et al.*, 1997a). Recently, OPG is reported to be approximately 25% higher in exercising young women compared to their sedentary counterparts (West *et al.*, 2008), although others report no effect of training status on OPG (Herrmann and Herrmann, 2004).

Several studies report that strenuous, acute exercise is associated with an increase in bone resorption markers that is accompanied by no increase or a less marked response in bone formation markers (Kerschan-Schindl *et al.*, 2009; Herrmann *et al.*, 2007; Maïmoun *et al.*, 2006; Guillemant *et al.*, 2004). As athletes regularly perform bouts of high intensity exercise, an increase in osteoclastic activity with a concomitant increase in activity of bone forming osteoblasts might provide a mechanism by which endurance training results in negative alterations in BMD.

Despite these studies, little is known about whether endurance-training alters the bone metabolic response to an acute bout of exercise. Only two studies have been performed in which volunteers of different training status have undergone the same acute exercise protocol (Nishiyama *et al.*, 1988; Herrmann *et al.*, 2007). Nishiyama *et al.* (1988) showed higher OC concentrations in male athletes compared with sedentary men 1 h after a 30 min treadmill run, although the magnitude of this difference was similar to that seen in basal concentrations and no other differences were observed. Additionally, no markers of bone resorption were measured and the exercise load was light (43 to 52% effort), which limits the interpretation of these findings in relation to bone turnover. In contrast, Herrmann *et al.* (2007), studied markers of both bone resorption and formation in male athletes and male sedentary controls performing 60 min of cycling using highly standardised exercise intensities. However, no statistical comparison between the two groups was made and the authors do not report

standardising the time of day or the nutritional status (*e.g.* fasted or fed) of subjects when collecting samples, both of which are known to affect bone marker concentrations, particularly β-CTX (Wichers *et al.*, 1999; Clowes *et al.*, 2002a; Henriksen *et al.*, 2003).

The aim of the present study was to examine the bone metabolic response to an acute bout of strenuous running exercise in recreationally-active (RA) and endurance-trained (ET) male runners.

4.2 Materials and Methods

4.2.1 Subjects

Eleven recreationally-active men and 11 endurance-trained male runners were recruited into the study to participate in an exhaustive exercise trial, forming a recreationally-active (RA) and an endurance-trained (ET) group. A further 11 recreationally-active men, matched with the RA group for aerobic fitness, were recruited to act as a non-exercising control group (CON). Subjects provided written informed consent. Subjects were included if they were non-smokers, had not suffered a bone fracture in the previous 12 months, were free from musculoskeletal injury, and were not taking any medication or suffering from any condition known to affect bone metabolism. Compliance with these inclusion criteria was confirmed from a medical screening questionnaire and a medical examination. Subjects were considered recreationally-active if they performed 2-3 exercise sessions per week including at least one run, but performed ≤ 2 h·week^{-1} of exercise and were not involved in an organised training programme (Table 4.1). Subjects in the ET group were required to have been running for a minimum of two years without a significant break and to have completed a measured 10 km distance in <40 min within the previous four weeks.

4.2.2 Overview of exercise intervention

On Day 4, RA and ET subjects completed an exercise protocol consisting of 60 min at 65% VO_{2max} followed by intermittent, exhaustive running at 70% VO_{2max} separated by a 15 min rest (Figure 4.1). Subjects in the CON group did not perform the exercise intervention.

4.2.3 Preliminary measures

Medical Screening

Subjects underwent a full medical examination as described in Section 3.2.1.

Dietary Analysis

Subjects completed a three-day food diary which was subsequently analysed as described in Section 3.2.2.

Determination of the association between oxygen uptake and running velocity during level (0° gradient) treadmill running and of maximal rate of oxygen uptake.

The association between VO_2 and velocity during level running was determined as described in Section 3.2.3. Maximal rate of oxygen uptake was determined as described in Section 3.2.4. The results of the two tests were used to estimate the treadmill velocity corresponding to 50, 65 and 70% VO_{2max} during level running based on the regression line of VO_2 and treadmill velocity.

Experimental dietary provision

Estimates of body composition (fat-free mass [FFM] and percentage body fat) were derived from an indirect measure of total body water using the bioelectrical impedance technique (BodyStat 500, Isle of Man, UK). A diet consisting of 8 g carbohydrate (CHO)·kg FFM^{-1}·day^{-1} and isocaloric with their habitual diet was designed for each subject based on individual dietary habits. Subjects were provided with three menus that were administered in a three-day cyclic order (Menu A on Days 1, 4 and 7, Menu B on Days 2 and 5, and Menu C on Days 3 and 6). Details of menus for individual subjects can be found in Appendix A.

4.2.4 Trial Procedures

Day 4 (RA and ET Groups only)

RA and ET subjects arrived at the laboratory at 0730 h after an overnight fast from 2100 h, provided a second void urine sample and had their nude body mass measured. Subsequently, subjects adopted a semi-recumbent position on a bed, and placed their left hand into a hand warming unit (Medical Physics, Nottingham, UK) where it remained for 20 min. A cannula (18GA 1.2x45 mm, Becton Dickinson, USA) was inserted into a vein in the back of the hand, secured and connected to a three-way tap (Connecta, Becton Dickinson, USA). The cannula was kept patent with an isotonic saline solution (0.9% NaCl). The hand was then returned to the hand warming unit for a further 20 min before a baseline (BASE) blood sample was collected for the measurement of all biochemical markers. During the experiment, the temperature inside the warming unit was held constant at 55°C to 60°C.

Following a 5 min warm-up at 50% VO_{2max} and a further 5 min for volitional stretching, subjects completed a 60 min run on a motorised treadmill at 65% of VO_{2max} (fixed duration exercise; FD). This was followed by a 15 min seated rest. Subsequently, subjects resumed exercise at 70% VO_{2max} and were instructed to run to volitional exhaustion at which point they rested for a further 5 min before resuming exercise. This work-rest pattern was repeated until subjects were not able to complete 5 min of continuous running at which rest breaks were reduced to 1 min. This new work-rest pattern was repeated until subjects were not able to complete 2 min of continuous running, at which point exercise was terminated. This work-rest protocol will be referred to as intermittent, exhaustive exercise (IEE).

Samples of expired air were collected for 60 sec after 5 min of FD and after the first 5 min of IEE and were analysed as described in Section 3.3.1.

Blood samples were collected after 20 (FD20), 40 (FD40), and 60 (FD60) min of FD, and again at the end of IEE (EE) for measurement of glucose, lactate, OPG, PTH, ACa and PO_4 (Figure 4.1). Heart rate was monitored continuously throughout the test and consumption of water was allowed *ad libitum*. On completion of IEE, subjects again adopted a semi-recumbent position and returned their

hand to the hand warming unit where it remained while they rested for 2 h. Further blood samples were drawn at 0.5 h (R0.5), 1 h (R1.0), 1.5 h (R1.5), and 2 h (R2.0) of recovery (Figure 4.1). Subjects remained fasted until the final blood sample was drawn after 2 h of recovery. No food or drink (except plain water) was consumed until the final blood sample was collected.

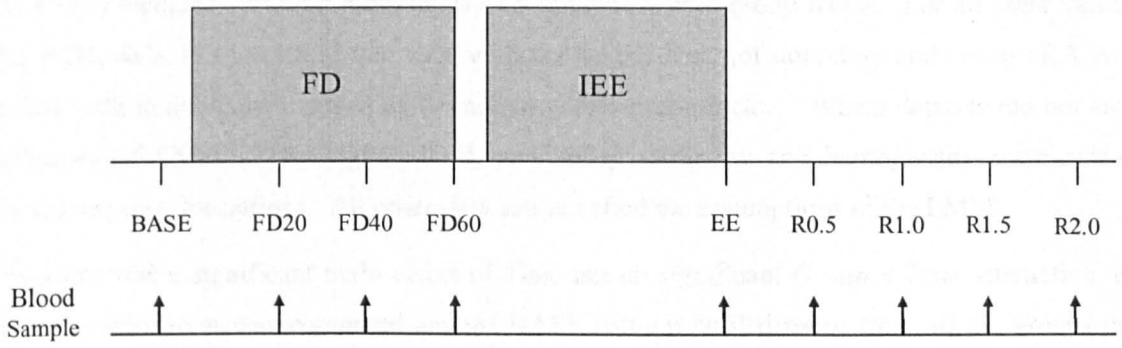

Figure 4.1. Outline of exercise protocol and blood sampling schedule on Day 4 of the experimental condition for the recreationally-active (RA) and endurance-trained (ET) groups. Subjects remained fasted until after the final blood sample was drawn at 2 h post-exercise (R2.0). Grey boxes denote exercise; FD – 60 min at 65% VO_{2max}; IEE – intermittent exhaustive exercise at 70% VO_{2max}.

Day 4 (CON group only)

After fasting from 2100 h the previous night, subjects arrived at the laboratory at 0730 h, provided a second void urine sample and had their nude body mass measured. A blood sample was then drawn by venepuncture for the measurement of all biochemical markers. In order to replicate the eating patterns of the RA and ET groups on Day 4, subjects in the CON group were asked to refrain from consuming any food until 1400 h.

4.2.5 Biochemical analysis

Blood samples drawn at BASE on Day 4 and on the four follow-up days (FU1 – FU4) were analysed for plasma β-CTX, P1NP, bone ALP, OPG, PTH, ACa and PO_4. All other blood samples drawn on Day 4 were analysed for OPG, PTH, ACa and PO_4 only. All urine samples were analysed for fPYD, fDPD and Cr.

4.2.6 Statistical Analysis

All data are presented as mean ± 1SD unless otherwise stated. Statistical significance was accepted at $P < 0.05$. One-way analysis of variance (ANOVA) was used to compare VO_{2max}, habitual and experimental dietary variables, and baseline biochemistry between the RA, ET and CON groups, with Newman Keuls *post hoc* tests performed where appropriate. Student's t-tests for unpaired data were used to compare variables relating to exercise performance between the RA and ET groups.

Paired samples t-tests were used to compare habitual with experimental dietary data within each of the three groups.

Where there were data for all three groups (plasma β-CTX, P1NP, bone ALP, fPYD, fDPD, Cr and body mass), data were analysed using a LMM, with the factors *Time* (of sampling) and *Group* (RA *vs* ET *vs* CON) included and with individuals as a random, within-group factor. For all other variables (OPG, PTH, ACa, PO_4), a LMM was used with the factors *Time* (of sampling) and *Group* (RA *vs* ET) included, with individuals included as a random, within-group factor. Where datasets did not satisfy assumptions of LMM (P1NP, OPG, PTH, and ACa), normality and homogeneity were achieved following log transformations. All other data sets satisfied the assumptions of the LMM.

Where there was a significant main effect of *Time* but no significant *Group* x *Time* interaction, each subsequent time point was compared against BASE using a pooled mean from all the groups using Dunnett's test with BASE as the 'Control'. When the *Group* x *Time* interaction was significant, within each group, each subsequent time point was compared against BASE using Dunnett's test with BASE as the '*Control*' and groups were compared to each other at all time points using the SNK test. In order to examine the overall effect of exercise on bone turnover markers, the area under the curve with respect to increase (AUC_I) described by Pruessner *et al.* (2003) was calculated, derived from the trapezoid formula (Reinhardt and Soeder, 2001). The AUC_I for the three groups were then compared with a one-way ANOVA and the SNK test for *post hoc* analyses.

Pearson's correlation coefficient was calculated between the percentage changes in PO_4 and PTH from BASE to FD20 in the RA and ET groups.

4.3 Results

4.3.1 Subject characteristics

Subject characteristics in the RA, ET and CON groups are shown in Table 4.1. One ET subject withdrew from the study due to the re-occurrence of a previous injury and one CON subject withdrew but did not provide a reason. Therefore, final statistical analyses were performed on 11 RA, 10 ET and 10 CON subjects.

Table 4.1. Subject characteristics of the recreationally-active (RA) endurance-trained (ET) and control (CON) groups.

	RA	ET	CON
n	11	10	10
Age (yr)	30 ± 3	31 ± 3 [e]	26 ± 3 [a]
Height (m)	1.84 ± 0.06	1.73 ± 0.06 [c, d]	1.79 ± 0.05
Body Mass (kg)	84.4 ± 8.4	69.8 ± 6.6 [b, d]	77.1 ± 7.0 [b]
BMI ($kg \cdot m^{-2}$)	25.0 ± 2.9	23.3 ± 1.6	24.0 ± 2.2
VO_{2max} ($ml \cdot min^{-1} \cdot kg^{-1}$)	55.5 ± 6.8	67.9 ± 6.1 [c, f]	53.7 ± 3.0
Physical activity ($h \cdot wk^{-1}$)	1.4 ± 0.4	4.5 ± 1.3 [c, f]	1.3 ± 0.5

Values are mean ± 1SD. [a] different ($P < 0.05$) from RA; [b] different ($P < 0.01$) from RA; [c] different ($P < 0.001$) from RA; [d] different ($P < 0.05$) from CON; [e] different ($P < 0.01$) from CON; [f] different ($P < 0.001$) from CON.

4.3.2 Dietary analysis and experimental dietary provision

Dietary analysis: Energy intake, macronutrient composition and dietary calcium from food diary analysis are shown in Table 4.2. ET subjects reported consuming a greater ($P < 0.05$) quantity of carbohydrate (CHO) relative to fat-free mass than both RA and CON but there were no other differences between the three groups for any other habitual dietary variable (Table 4.2). Daily calcium intake reported by all subjects exceeded 700 $mg \cdot day^{-1}$.

Table 4.2. Habitual energy intake, macronutrient composition and dietary calcium content of the recreationally-active (RA), endurance-trained (ET) and control (CON) groups.

	RA	ET	CON
Energy (MJ)	11.0 ± 1.5	11.6 ± 2.6	10.5 ± 1.9
CHO (g)	324 ± 59	383 ± 65	328 ± 70
CHO (g·kg FFM^{-1})	5.4 ± 0.9	6.7 ± 1.3 [a d]	5.4 ± 0.8
CHO (% of total energy)	47.3 ± 6.1	53.2 ± 6.5	49.4 ± 5.7
Fat (% of total energy)	30.9 ± 4.5	27.2 ± 7.0	30.2 ± 6.5
Protein (% of total energy)	18.1 ± 2.3	17.8 ± 3.5	17.4 ± 2.5
Calcium (mg·day^{-1})	1210	1184	1044 ± 264
	(range, 852-1823)	(range, 708-1963)	(range, 719-1556)

Values are mean ± 1SD unless otherwise stated. CHO, carbohydrate; FFM, fat-free mass. [a] different ($P < 0.05$) from RA; [d] different ($P < 0.05$) from CON.

Experimental dietary provision: Energy intake, macronutrient composition and dietary calcium in experimental diets are shown in Table 4.3. There was no significant difference between the energy content of experimental and habitual diets in any group and there were no significant differences between the three groups for any experimental dietary variable (Table 4.3).

Table 4.3. Energy content and macronutrient composition of the experimental diets consumed by the recreationally-active (RA), endurance-trained (ET) and control (CON) groups during the experimental condition.

	RA	ET	CON
Energy (MJ)	11.4 ± 1.6	12.0 ± 1.6	10.8 ± 1.4
CHO (g)	483 ± 35 ***	456 ± 36	481 ± 62 ***
CHO (g·kg FFM^{-1})	7.99 ± 0.02 ***	7.98 ± 0.05 **	7.99 ± 0.05 ***
CHO (% of total energy)	67.9 ± 6.1 ***	61.4 ± 9.2 **	64.6 ± 5.2 ***
Fat (% of total energy)	17.0 ± 6.0 ***	23.7 ± 8.9	20.9 ± 5.4 **
Protein (% of total energy)	15.1 ± 0.6 *	14.9 ± 1.3 *	14.6 ± 0.8 **
Calcium (mg·day^{-1})	1126 ± 307	1168 ± 207	1187 ± 243

Values are mean ± 1SD. CHO, carbohydrate; FFM, fat-free mass; * different ($P < 0.05$) from habitual diet; ** different ($P < 0.01$) from habitual diet; *** different ($P < 0.001$) from habitual diet.

4.3.3 Baseline biochemistry

There were no significant differences in baseline values for biochemical markers of bone turnover between the RA and ET groups (Table 4.4). Cr concentrations were significantly higher in CON compared with both RA ($P < 0.05$) and ET ($P < 0.001$), fDPD/Cr concentrations in ET and CON were significantly lower ($P < 0.05$) than in RA. β-CTX was 36% and 23% higher in CON compared with

ET and RA, but these differences were not significant (P = 0.150). P1NP was approximately 25% lower in ET compared with RA and CON although, again, this difference was not significant (P = 0.071). OPG in CON was 25% and 38% higher than RA and ET but this difference was also not significant (P = 0.087).

Table 4.4. Baseline biochemistry of the recreationally-active (RA), endurance-trained (ET) and control (CON) groups.

Variable	RA	ET	CON	P Value (one-way ANOVA)
β-CTX ($ug \cdot mL^{-1}$)	0.40 ± 0.12	0.36 ± 0.13	0.49 ± 0.20	0.150
P1NP ($ug \cdot mL^{-1}$)	49 ± 15	38 ± 13	52 ± 15	0.071
Bone ALP ($U \cdot L^{-1}$)	20 ± 8	19 ± 8	24 ± 6	0.258
Cr ($mmol \cdot L^{-1}$)	6.4 ± 4.9	4.9 ± 1.9 [f]	14.6 ± 7.0 [b]	P < 0.001
fPYD/Cr ($nmol \cdot mmol\ Cr^{-1}$)	19.4 ± 5.6	16.1 ± 4.4	17.4 ± 3.4	0.267
fDPD/Cr ($nmol \cdot mmol\ Cr^{-1}$)	4.6 ± 1.1	3.5 ± 0.7 [a]	3.9 ± 0.6 [a]	0.016
OPG ($pmol \cdot L^{-1}$)	2.9 ± 0.9	3.2 ± 1.5	4.0 ± 1.0	0.087
PTH ($pmol \cdot L^{-1}$)	2.2 ± 0.5	2.7 ± 1.2	2.6 ± 0.7	0.278
ACa ($mmol \cdot L^{-1}$)	2.43 ± 0.05	2.44 ± 0.09	2.38 ± 0.09	0.296
PO_4 ($mmol \cdot L^{-1}$)	1.06 ± 0.16	1.07 ± 0.10	1.04 ± 0.12	0.892

Values are mean ± 1SD. [a] different (P < 0.05) from RA; [b] different (P < 0.01) from RA; [f] different (P < 0.001) from CON.

4.3.4 Body Mass

Body mass did not change significantly from Day 4 to Day 8 in any of the three groups (Figure 4.2).

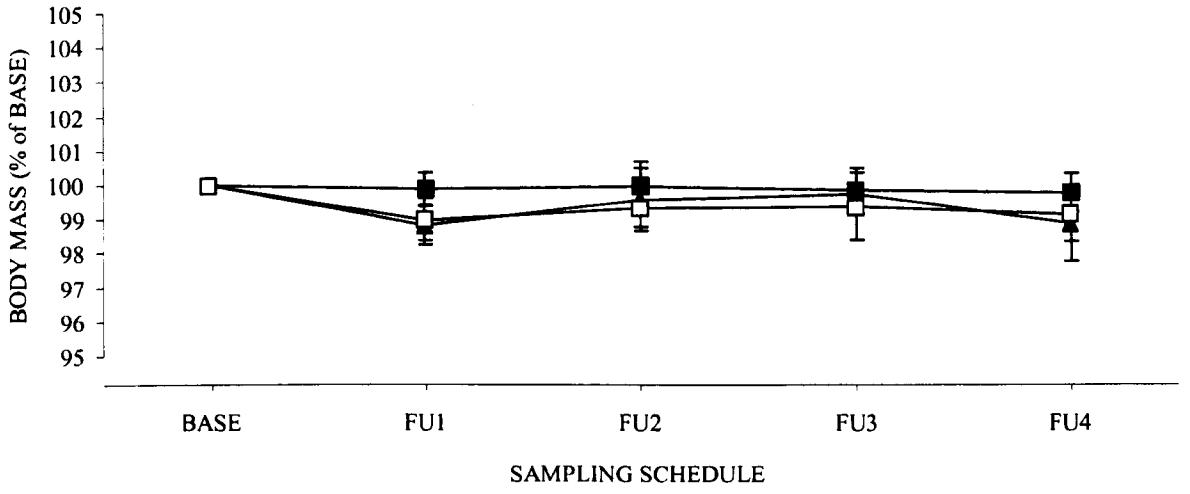

Figure 4.2. Percentage change in body mass from baseline (BASE) on the four follow-up days (FU1 – FU4) in the recreationally-active (RA – open squares), endurance-trained (ET – filled triangles) and control (CON – filled squares) groups. Values are mean ± 1SD.

4.3.5 Exercise variables and performance

Mean running speed was higher in ET compared with RA during both FD ($P < 0.001$) and IEE ($P < 0.001$) (Table 4.5) but there were no significant differences in VO_2 or RER. Total exercise duration tended to be greater in the ET group than the RA group (134 ± 14 *vs* 116 ± 27 min), although this difference did not reach the assigned level of significance ($P = 0.079$). As the ET group ran significantly faster than the RA group during both FD and IEE, the total distance covered by ET was approximately 50% greater than that by RA (29.1 ± 3.8 *vs* 19.8 ± 3.5 km, $P < 0.001$).

Table 4.5. Variables relating to exercise in the recreationally-active (RA) and endurance-trained (ET) groups.

Variable	RA	ET	P Value t-test for unpaired data
FD			
Speed ($km \cdot h^{-1}$)	10.0 ± 1.3	12.6 ± 0.5	<0.001
% VO_{2max}	62 ± 4	61 ± 3	0.300
RER	0.900 ± 0.034	0.880 ± 0.033	0.185
IEE			
Speed ($km \cdot h^{-1}$)	10.8 ± 1.4	13.4 ± 0.5	<0.001
% VO_{2max}	68.8 ± 4.4	67.1 ± 3.5	0.370
RER	0.898 ± 0.043	0.879 ± 0.036	0.313
Time (min)	56.0 ± 26.8	73.7 ± 14.3	0.079
Total Time (min)	116 ± 27	134 ± 14	0.079
Total distance (km)	19.8 ± 3.5	29.1 ± 3.8	<0.001

Values are mean ± 1SD. FD, fixed duration exercise (60 min at 65% VO_{2max}); IEE, intermittent, exhaustive exercise at 70% VO_{2max}.

4.3.6 Glucose, lactate and insulin

Glucose: There was a significant main effect of *Time* ($P < 0.001$) but no significant *Group* x *Time* interaction ($P = 0.754$) for glucose concentrations. Pooled, mean glucose concentrations were increased ($P < 0.001$) from BASE in both RA and ET throughout fixed duration exercise (Figure 4.3, Panel A). At EE blood glucose was lower ($P < 0.001$) compared with BASE in both RA (3.6 ± 0.9 $mmol \cdot L^{-1}$) and ET (3.6 ± 0.4 $mmol \cdot L^{-1}$) and remained so until 2 h post-exercise. The glucose response to exercise was not significantly different between RA and ET.

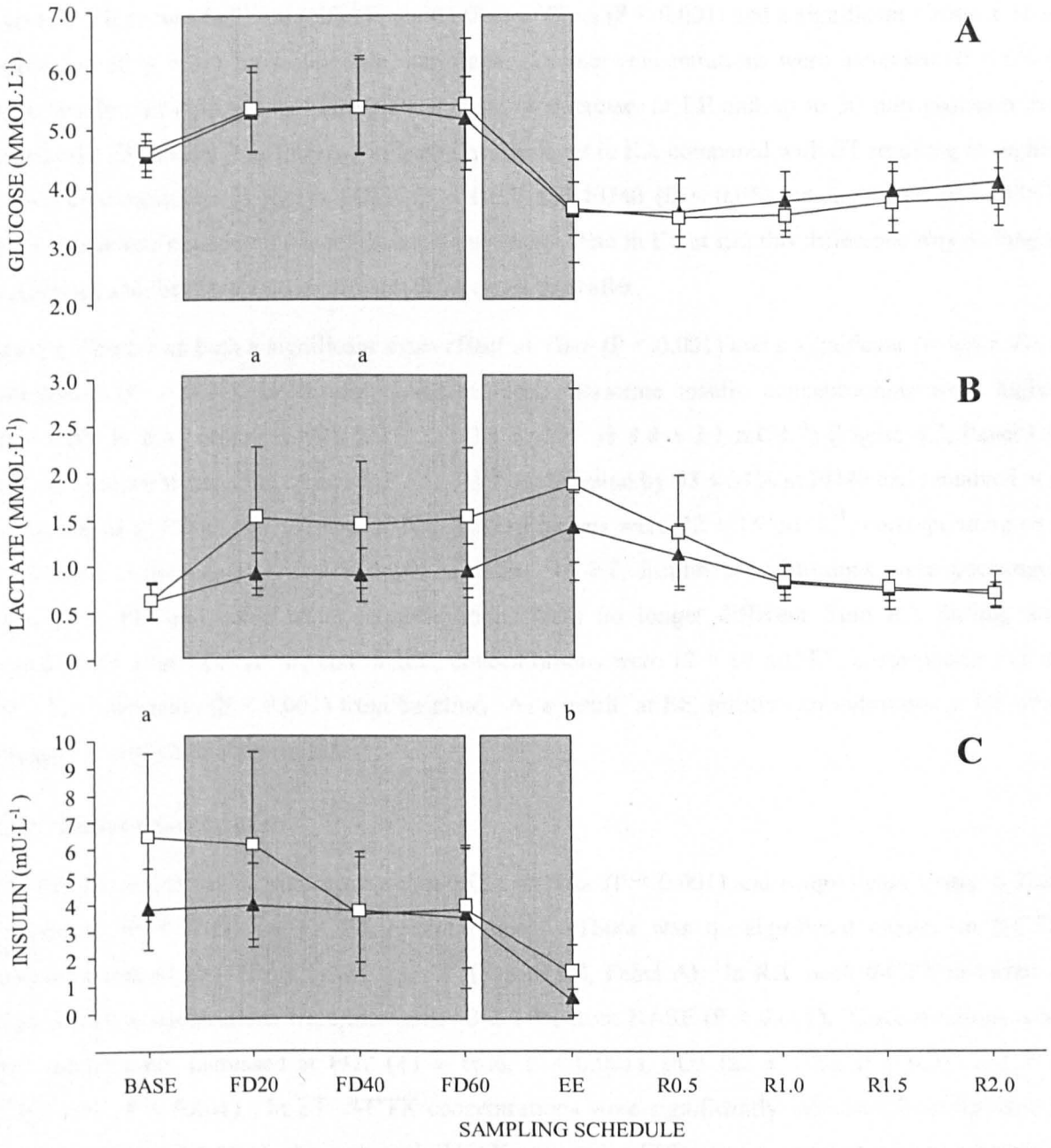

Figure 4.3. Effect of exhaustive exercise on blood glucose (Panel A), lactate (Panel B) and insulin (Panel C) in the recreationally-active (RA – open squares) and endurance-trained (ET – filled triangles) groups. Values are mean ± 1SD. Pooled, glucose concentrations were significantly higher than BASE during exercise and significantly lower following exercise. Pooled lactate concentrations were higher than BASE during exercise and up to 30 min post-exercise. Insulin concentrations in RA were significantly lower than BASE from FD40 up to EE while in ET, concentrations were significantly lower than BASE only at EE. [a] different ($P < 0.05$) from RA; [b] different ($P < 0.01$) from RA.

Lactate: There was both a significant main effect of *Time* ($P < 0.001$) and a significant *Group* x *Time* interaction ($P < 0.01$) for lactate concentrations. Lactate concentrations were increased ($P < 0.05$) from baseline in both groups during fixed duration exercise, at EE and up to 30 min post-exercise (Figure 4.3, Panel B). The increase in lactate was greater in RA compared with ET resulting in higher lactate concentrations in RA at FD20 ($P < 0.05$) and FD40 ($P < 0.05$) but not FD60 ($P = 0.06$). Although lactate concentrations remained higher in RA than in ET at EE, this difference was no longer significant and there were no significant differences thereafter.

Insulin: There was both a significant main effect of *Time* ($P < 0.001$) and a significant *Group* x *Time* interaction ($P < 0.01$) for insulin concentrations. Baseline insulin concentrations were higher ($P < 0.05$) in RA compared with ET (6.4 ± 3.1 mU·L^{-1} *vs* 3.8 ± 3.1 mU·L^{-1}) (Figure 4.3, Panel C). In RA, concentrations were reduced ($P < 0.01$) from baseline by $43 \pm 31\%$ at FD40 and remained at a similar level at FD60. At the end of IEE, concentrations were 22 ± 15 mU·L^{-1}, corresponding to a $78 \pm 15\%$ reduction ($P < 0.001$) from baseline. In ET, insulin concentrations were unchanged throughout FD and, as a result, concentrations were no longer different from RA during, and immediately after FD. At the end of IEE, concentrations were 12 ± 10 mU·L^{-1}, corresponding to an $88 \pm 10\%$ reduction ($P < 0.001$) from baseline. As a result, at EE, insulin concentrations in ET were lower ($P < 0.01$) than those in RA.

4.3.7 Bone turnover markers

β-CTX: There was both a significant main effect of *Time* ($P < 0.001$) and a significant *Group* x *Time* interaction ($P < 0.05$) for β-CTX concentrations. There was no significant change in β-CTX concentrations in CON from BASE to FU4 (Figure 4.4, Panel A). In RA, peak β-CTX occurred at FU1, where concentrations were increased $43 \pm 19\%$ from BASE ($P < 0.001$). Concentrations were also significantly increased at FU2 ($33 \pm 16\%$, $P < 0.001$), FU3 ($22 \pm 19\%$, $P < 0.01$) and FU4 ($30 \pm 19\%$, $P < 0.001$). In ET, β-CTX concentrations were significantly increased from baseline at FU1 ($23 \pm 27\%$, $P < 0.01$), although peak β-CTX occurred at FU2 when concentrations were increased by $46 \pm 31\%$ ($P < 0.001$). Concentrations remained significantly higher than BASE at FU3 ($32 \pm 33\%$, $P < 0.01$) and FU4 ($22 \pm 27\%$, $P < 0.05$). $AUC_{I(BASE - FU4)}$ for β-CTX was significantly greater ($P < 0.01$) in RA and ET compared with CON (Figure 4.5) but there was no significant difference between the RA and ET groups.

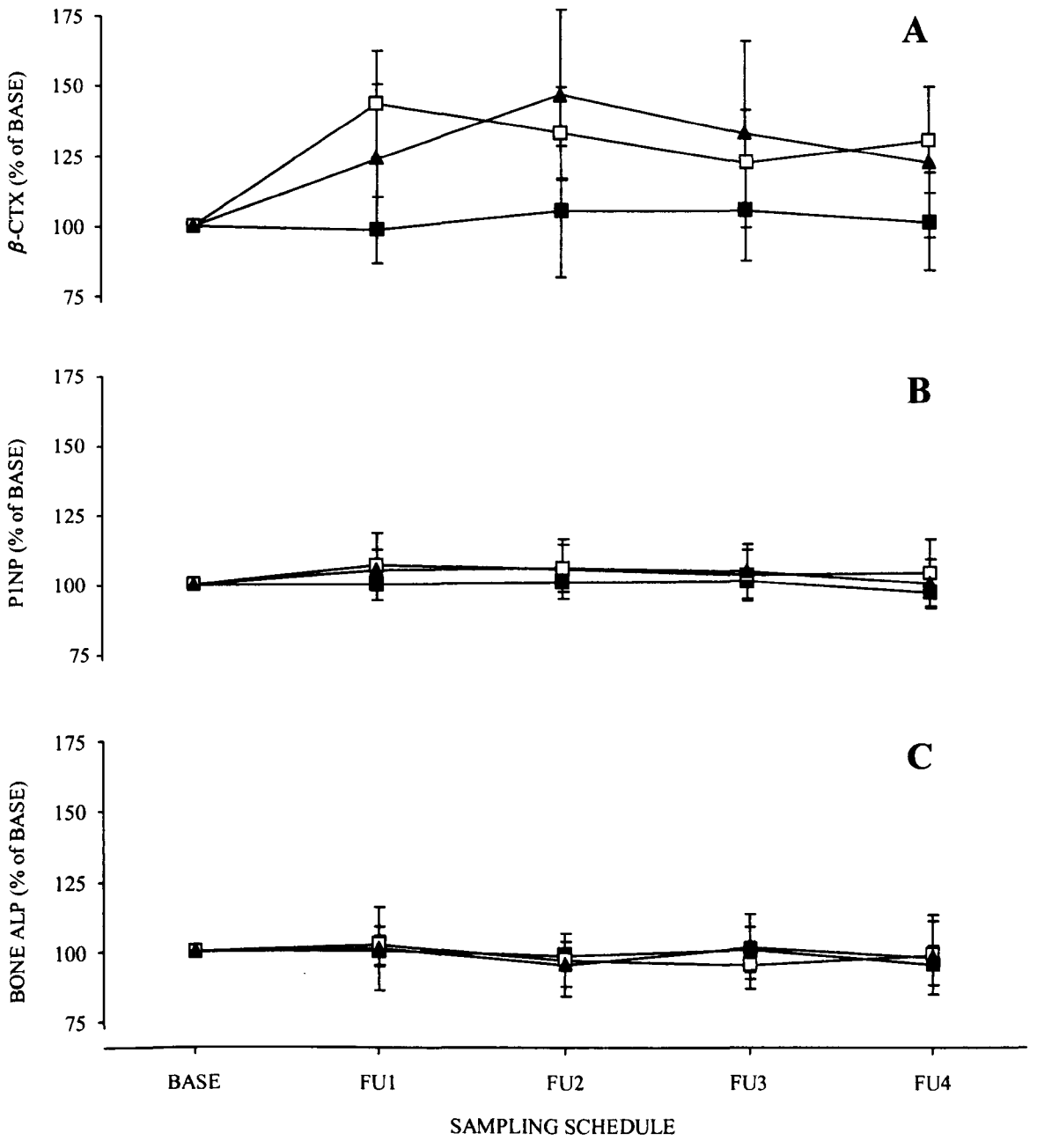

Figure 4.4. The percentage change in BASE concentrations of β-CTX (Panel A), P1NP (Panel B) and bone ALP (Panel C) on the four follow-up days (FU1 – FU4) in the recreationally-active (RA – open squares), endurance-trained (ET – filled triangles) and control (CON – filled squares) groups. Values are mean ± 1SD. β-CTX concentrations were unchanged in CON but significantly increased from BASE at FU1 – FU4 in both RA and ET.

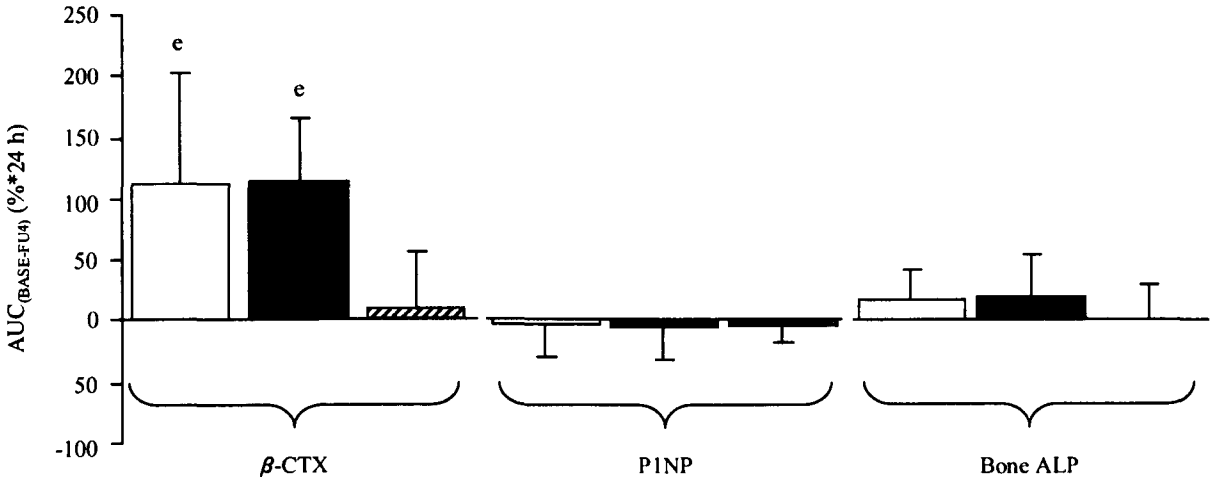

Figure 4.5. $AUC_{I(BASE - FU4)}$ analysis of β-CTX, P1NP (Panel B) and bone ALP in the RA (Open bars), ET (Solid bars) and CON (Hatched bars) groups. Values are mean ± 1SD. [e] different ($P < 0.01$) from CON.

P1NP: Both ANOVA and $AUC_{I(BASE - FU4)}$ analyses indicated that there was no significant effect of exercise on P1NP (Figure 4.4, Panel B and Figure 4.5).

Bone ALP: Both ANOVA and $AUC_{I(BASE - FU4)}$ analyses indicated that there was no significant effect of exercise on bone ALP concentrations (Figure 4.4, Panel C and Figure 4.5).

fPYD/Cr: There was a significant main effect of *Time* ($P < 0.05$) but no significant *Group* x *Time* interaction ($P = 0.352$) for fPYD/Cr concentrations. Pooled, mean concentrations were significantly reduced from BASE at FU2 ($P < 0.001$) where concentrations were reduced 13%, 4% and 11% in RA, ET and CON (Figure 4.6, Panel A). Concentrations were not significantly different from BASE thereafter.

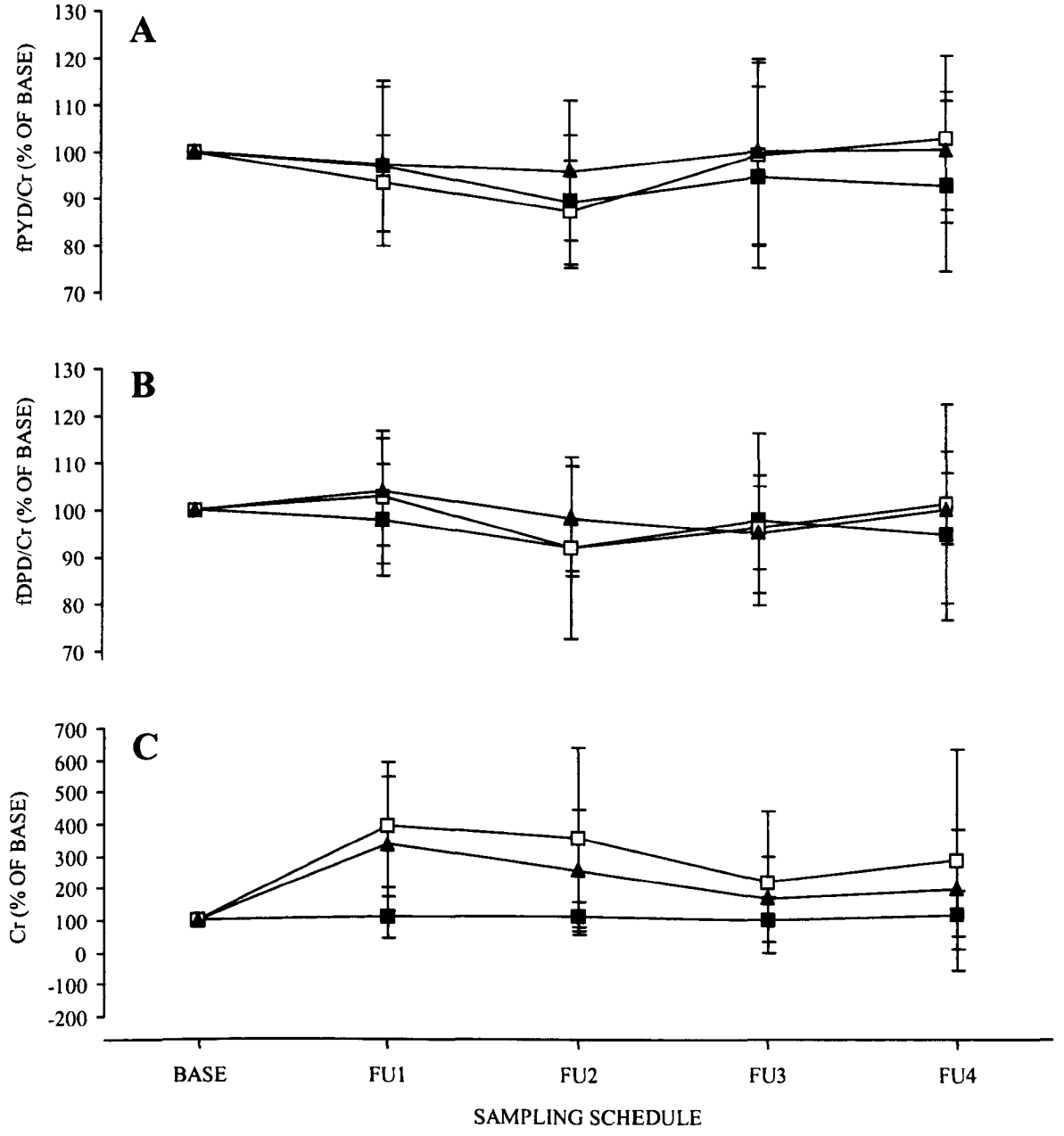

Figure 4.6. The percentage change in BASE concentrations of fPYD/Cr (Panel A), fDPD/Cr (Panel B) and Cr (Panel C) on the four follow-up days (FU1 – FU4) in the recreationally-active (RA – open squares), endurance-trained (ET – filled triangles) and control (CON – filled squares) groups. Values are mean ± 1SD. Pooled, fPYD/Cr and PDD/Cr concentrations were significantly lower than BASE at FU2 only. Cr concentrations were unchanged in CON but significantly higher than BASE at FU1 and FU2 in RA, and at FU1 only in ET.

fDPD/Cr: There was a significant main effect of *Time* ($P < 0.05$) but no significant *Group* x *Time* interaction ($P = 0.651$) for fDPD/Cr concentrations. Pooled, mean concentrations were significantly reduced from BASE at FU2 ($P < 0.01$) where they were reduced 8%, 2% and 8% in RA, ET and CON (Figure 4.6, Panel B). Concentrations were not significantly different from BASE thereafter.

Cr: There was both a significant main effect of *Time* ($P < 0.001$) and a significant *Group* x *Time* interaction ($P < 0.05$) for Cr concentrations. There was no significant change in Cr concentrations in CON from BASE to FU4 (Figure 4.6, Panel C). In RA, Cr concentrations were significantly increased from BASE at FU1 (397%, $P < 0.001$) and FU2 (357%, $P < 0.01$) but were not different from BASE at FU3 and FU4. In ET, Cr concentrations were significantly increased from BASE at FU1 (336%, $P < 0.001$) but were not significantly different from BASE thereafter. There was considerable variability in changes in Cr between individual subjects with exercise in both the RA (Figure 4.7, Panel A) and ET (Figure 4.7, Panel B) groups. Although the general pattern of change was similar between subjects, with peak concentrations occurring at either FU1 or FU2, there was a high degree of individuality in the magnitude of these changes. Cr concentrations in the CON group also varied across the five measurement days with some subjects showing decreases of up to 60% from BASE while others showing increases in excess of 200% (Figure 4.7, Panel C).

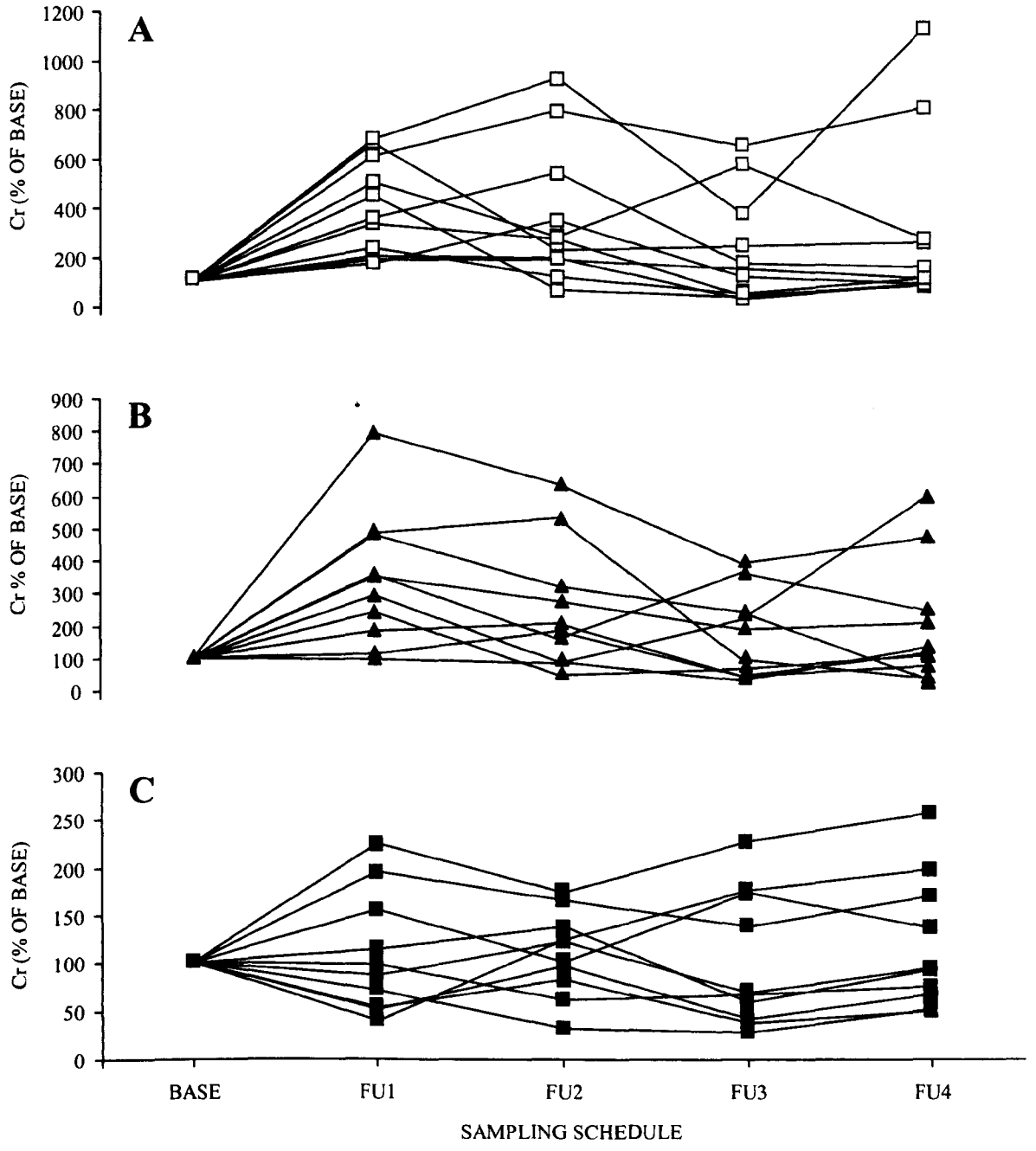

Figure 4.7. The percentage change in BASE concentrations of Cr in the RA (Panel A), ET (Panel B) and CON (Panel C) on the four follow-up days (FU1 – FU4). Different scales are used for clarity.

4.3.8 OPG

There was a significant main effect of *Time* ($P < 0.001$) for OPG concentrations and a tendency towards a *Group* x *Time* interaction but this did not reach statistical significance ($P = 0.082$). Pooled, mean concentrations increased during FD and were significantly ($P < 0.001$) higher than BASE at FD60, increased by 20 ± 16% and 27 ± 38% in RA and ET (Figure 4.8). At EE, OPG concentrations in RA and ET were 28 ± 23% and 40 ± 47% higher ($P < 0.001$) than at BASE and remained significantly elevated for 2 h post-exercise ($P < 0.001$). Concentrations were reduced

slightly from EE during the first hour post-exercise before increasing again at 2 h post-exercise. OPG concentrations were still significantly elevated from BASE in ET (16 ± 36%) and RA (18 ± 9%) at FU1 ($P < 0.001$) but there were no differences from BASE thereafter.

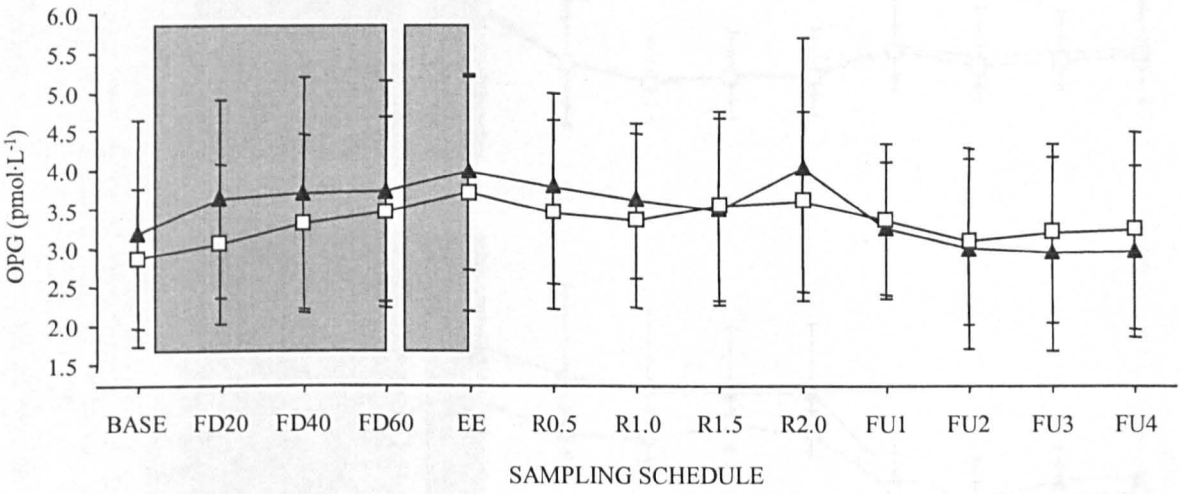

Figure 4.8. OPG concentrations before (BASE), during (FD20-FD60) and for 2 h after (EE-R2.0) exhaustive exercise, and on the four follow-up days (FU1 – FU4) in the recreationally-active (RA – open squares) and endurance-trained (ET – filled triangles) groups. Values are mean ± 1SD. Pooled, OPG concentrations were significantly higher than BASE during exercise and remained so up to FU1.

4.3.9 Calcium metabolism

PTH: There was a significant effect of *Time* ($P < 0.001$) but no *Group* x *Time* interaction ($P = 0.464$) for PTH concentrations. Pooled, mean concentrations were significantly ($P < 0.001$) increased from BASE at FD20 by 229 ± 66% and 157 ± 43% in RA and ET (Figure 4.9, Panel A). PTH remained significantly elevated from BASE at FD60 and at EE in both groups ($P < 0.001$) although concentrations were reduced from those in FD to 166 ± 67% and 137 ± 50% of BASE in RA and ET. Following 30 min of recovery, PTH concentrations had returned to BASE levels in both groups before decreasing significantly at R1.0, R1.5 and R2.0 ($P < 0.001$). The lowest mean concentrations of PTH were observed at 60 min of recovery in both RA (1.8 ± 0.5 pmol·L^{-1}) and ET (1.9 ± 1.1 pmol·L^{-1}), with values being reduced by 17% and 30% from BASE. PTH concentrations were similar to BASE on the four follow-up days and there were no significant differences in the pattern of change in PTH between the groups.

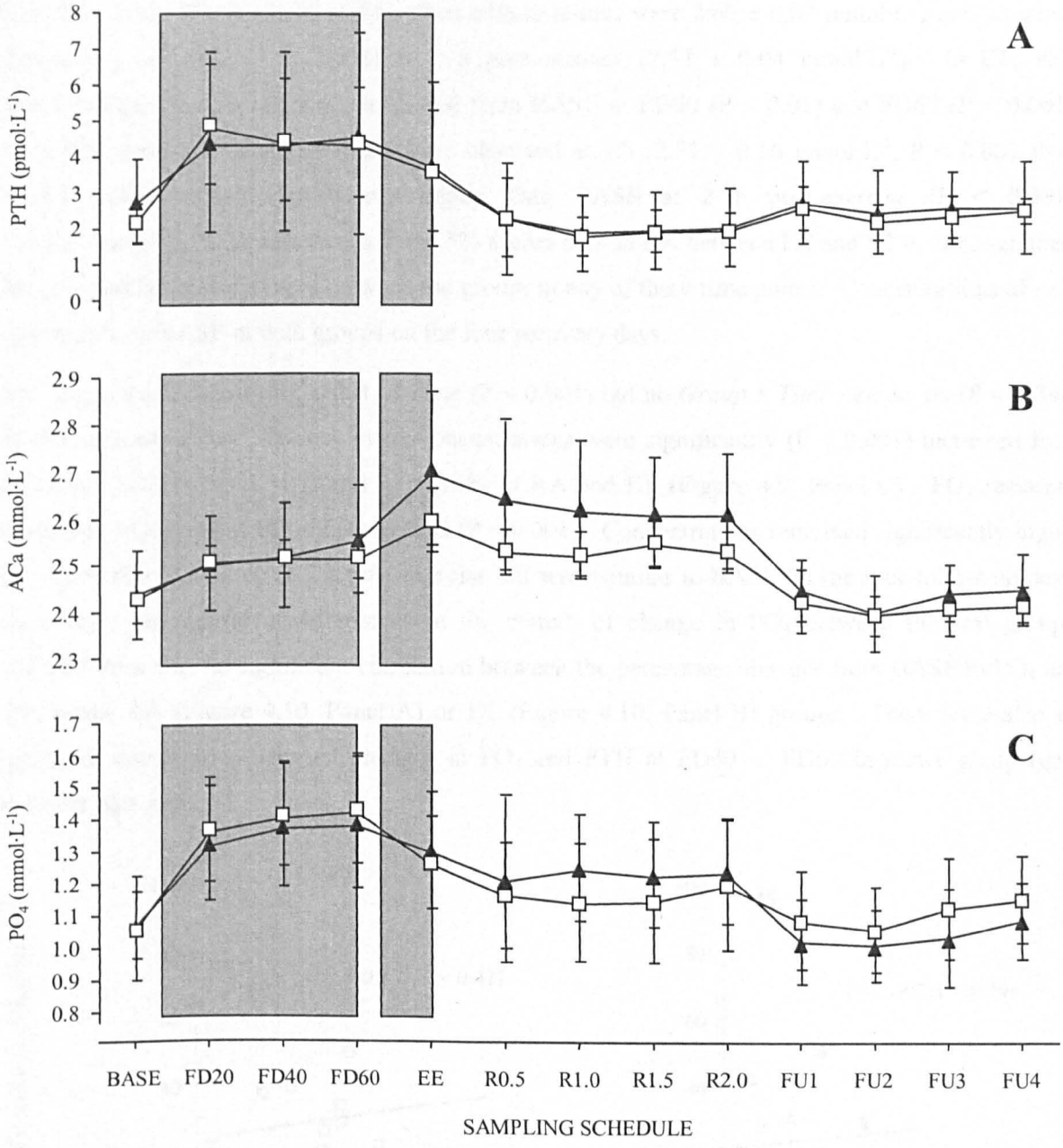

Figure 4.9. PTH (Panel A), ACa (Panel B) and PO_4 (Panel C) concentrations before (BASE), during (FD20-FD60) and for 2 h after (EE-R2.0) exhaustive exercise, and on the four follow-up days (FU1 – FU4) in the recreationally-active (RA – open squares) and endurance-trained (ET – filled triangles) groups. Values are mean ± 1SD. Pooled, PTH concentrations were significantly higher than BASE throughout exercise and significantly lower than BASE at R1.0, R1.5 and R2.0. In RA, ACa concentrations were significantly higher than BASE throughout exercise and up to 2 h post exercise while in ET, concentrations were significantly higher than BASE from FD40 up to 2 h post exercise. Pooled, PO_4 concentrations were significantly ($P < 0.001$) higher than BASE throughout exercise and up to 2 h post-exercise.

ACa: There was both a significant main effect of *Time* ($P < 0.001$) and a significant *Group* x *Time* interaction ($P < 0.001$) for ACa concentrations. In RA, the concentration of ACa was increased ($P < 0.01$) at FD20 compared with BASE, and remained elevated ($P < 0.01$) at FD60 (Figure 4.9,

Panel B). Peak ACa occurred at EE, when concentrations were 2.60 ± 0.07 mmol·L^{-1}, and remained significantly elevated ($P < 0.001$) at 2 h post-exercise (2.53 ± 0.04 mmol·L^{-1}). In ET, ACa concentrations were significantly increased from BASE at FD40 ($P < 0.01$) and FD60 ($P < 0.001$). As in RA, peak ACa concentrations were observed at EE (2.71 ± 0.16 mmol·L^{-1}, $P < 0.001$ from BASE) and remained significantly higher than BASE at 2 h post-exercise ($P < 0.001$). Concentrations in ET were between 2 and 5% higher than in RA between EE and R2.0, although there were no significant differences between the groups at any of these time points. Concentrations of ACa were similar to BASE in both groups on the four recovery days.

PO_4: There was a significant effect of *Time* ($P < 0.001$) but no *Group* x *Time* interaction ($P = 0.398$) for PO_4 concentrations. Pooled, mean concentrations were significantly ($P < 0.001$) increased from BASE at FD20 by 30 ± 12% and 24 ± 15% in RA and ET (Figure 4.9, Panel C). PO_4 remained elevated at FD60 and at EE in both groups ($P < 0.001$). Concentrations remained significantly higher ($P < 0.05$) than BASE up to 2 h post-exercise but were similar to BASE on the four follow-up days. There were no significant differences in the pattern of change in PO_4 between the two groups. At FD20 there was no significant correlation between the percentage changes from BASE in PO_4 and PTH in the RA (Figure 4.10, Panel A) or ET (Figure 4.10, Panel B) groups. There were also no significant correlations between changes in PO_4 and PTH at FD40 or FD60 in either group (data shown in Appendix B).

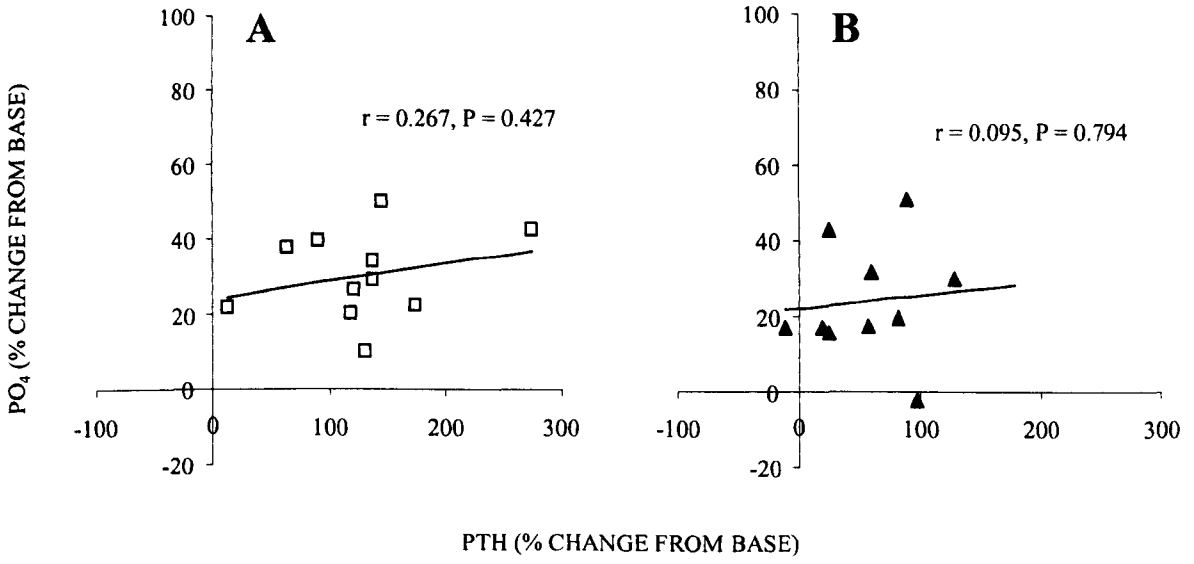

Figure 4.10. Relationship between the percentage changes in PO_4 and PTH concentrations measured from BASE to FD20 in the RA (Panel A) and ET (Panel B) groups.

4.4 Discussion

The main findings from this study are that 1) compared to non-exercising controls, acute, exhaustive, running is associated with an increase in blood-base markers of bone resorption but not bone formation in recreationally-active men, that is not altered by improved training status; 2) despite the increase in β-CTX, there was no effect of exhaustive running on Cr-corrected urinary free pyridinolines, although exercise did significantly increase urinary Cr concentrations; 3) exercise was associated with an increase in circulating OPG, with concentrations displaying a peak at the end of exercise and another at 2 h post-exercise; 4) PTH concentrations were increased after 20 min of running, remained increased up to the termination of exercise and returned to baseline by 30 min post-exercise and; 5) both ACa and PO_4 were significantly increased after 20 min of running and remained elevated up to 2 h post-exercise, with peak ACa and PO_4 concentrations occurring at the end of IEE and after 20 min of FD.

Exhaustive running was associated with a significant and sustained increase in bone resorption, as assessed by β-CTX, in RA and ET men. The 45% increase in β-CTX in the present study is similar to the increase reported by Guillemant *et al.* (2004) following 60 min of cycling at 80% VO_{2max} but smaller than the 75% increase reported by Kerschan-Schindl *et al.* (2009) immediately after a 246 km running race. A smaller increase (10-20%) immediately following 50 min of cycling at 115% of VT (Maïmoun *et al.*, 2006) and no increase at 3 h after a 60 min run at 70% of LT in young male athletes (Zittermann *et al.*, 2002) have also been reported. Herrmann *et al.* (2007) detected a significant increase in β-CTX at 3 h and 24 h following 60 min cycling at 110% of AT, although they did not standardise the time of day of exercise or the nutritional status of subjects prior to sample collection. Kerschan-Schindl *et al.* (2009) report a 40% increase in β-CTX the morning after a 246 km running race but they do not report specifically at what time, whilst they also did not control for the nutritional status of subjects prior to sample collection. Thus, the present study appears to be the first controlled investigation to show that β-CTX is elevated in response to exhaustive exercise and remains so for more than 24 h.

In contrast to plasma β-CTX, the urinary markers of bone resorption fPYD and fDPD showed no significant difference in their pattern of change compared to the non-exercising control group, with Cr-corrected concentrations decreasing on the second recovery day. There is little in the way of comparative literature on the effects of acute exercise on pyridinolines. Urinary fDPD is decreased in spot samples at 1 h (Ashizawa *et al.*, 1997) or at 3 days (Ashizawa *et al.*, 1998) following a strenuous bout of resistance exercise, whereas Brown *et al.* (1997) report no change in Cr-corrected fPYD between 1 and 9 days following a single bout of 50 eccentric single-leg knee extensor contractions. In contrast to the present study, however, Welsh *et al.* (1997) report a significant increase of 50% in both fPYD/Cr and fDPD/Cr in a 24 h urine sample collected the day after 30 min of walking at 60% of HR_{max}.

Given the lack of any increase in fPYD/Cr and fDPD/Cr in the present study and the nature of the exercise performed, the magnitude of the increase in pyridinolines in the study of Welsh *et al.* (1997) is perhaps surprising. While this difference might be due to the sampling methodology (24 h *vs* SMV), in the present study it is also possible that changes in Cr might have been an important confounding factor. The 3- to 4-fold increase in Cr seen in both exercising groups is greater in magnitude than that reported following much longer duration, lower intensity running (Nieman *et al.*, 2006) as well as with high intensity, endurance skiing (Refsum and Strömme, 1974). No previous studies appear to have measured increases in urinary Cr concentrations beyond 24 h post-exercise although it is interesting to note that Tosun *et al.* (2006), measured fDPD corrected for Cr in spot samples taken before and 24 h after exercise but choose not to analyse them due to high variability between samples. Unfortunately, they do not provide any further information as to the nature of this variability but it is possible that it was related to changes in Cr. The present study indicates that acute exhaustive, running is associated with a significant increase in Cr concentrations for 48 h after exercise and, although concentrations were not significantly different from BASE on FU3 and FU4, some subjects still showed increased Cr levels resulting in mean Cr concentrations of 165 to 286% of BASE levels.

Given the increase in β-CTX in this study, it is possible that the increase in Cr may have masked an increase in fPYD and fDPD when they were corrected for Cr concentrations, particularly at FU1 and FU2 when the increases in Cr were greatest. Although Welsh *et al.* (1997) do not report raw Cr data, moderate exercise has been shown not to increase urinary Cr levels (Plante and Houston, 1984). Unchanged Cr levels, therefore, might have made changes in pyridinolines more evident. This is the first study of acute exercise to measure both β-CTX and pyridinolines so there is no previous information as to how changes in pyridinolines reflect those of β-CTX with exercise. If the 50% increase in pyridinolines observed by Welsh *et al.* (1997) is a typical response to exercise, the magnitude of the increase in Cr in the present study would almost certainly be sufficient to mask such an increase.

That said, the increase in Cr concentrations with exercise seems unlikely to explain the significant reduction in fPYD and fDPD at FU2 as the decrease in the CON group was approximately the same as that in the RA group for fPYD (11% *vs* 13%) and fDPD (8% in both groups) and greater than that in the ET group (4% and 2% for fPYD and fDPD) despite no change in mean urinary Cr levels. Alternatively, the significant fall in fPYD/Cr and fDPD/Cr at FU2 might be explained by the feeding schedule. Concentrations of fPYD and fDPD are reduced following a 24 h fast (Talbott and Shapses, 1998) and, in the present study, all subjects fasted for a minimum of 17 h between the BASE and FU1 urine samples. Therefore, it is possible, although only speculative, that this period without food may, in part, explain the transient suppression of fPYD/Cr and fDPD/Cr concentrations.

Finally, despite no significant change in mean Cr concentrations in the CON group, the variability in the individual data appears to support the premise that day-to-day Cr excretion is not, as previously concluded (Folin, 1904), constant in a given individual.

Bone formation, as indicated by P1NP and bone ALP concentrations, was not altered by exhaustive running. Few studies have used P1NP as a bone formation marker (Miller *et al.*, 2005; Tosun *et al.*, 2006; Herrmann *et al.*, 2007), despite its preferred use in monitoring changes in bone formation in clinical settings (Dominguez Cabrera *et al.*, 1998). Tosun *et al.* (2006) reported no change in P1NP immediately following 30 min of treadmill walking, whereas Herrmann *et al.* (2007) observed consistent decreases in P1NP concentrations at 3 h and 24 h following 60 min of cycling at 75% of AT. Miller *et al.* (2005) showed no change in P1NP 24 h, 48 h and 72 h following 60 min of one-legged knee extensor exercise at 67% workload max. The lack of an effect of exercise on bone ALP is consistent with the data of Guillemant *et al.* (2004) who showed no change in bone ALP during, and up to 2 h after 60 min of strenuous cycling. When considered altogether, it appears that there is no sustained effect of acute exercise on bone formation, at least up to 4 days post-exercise.

The increase in bone resorption but not formation in the four days following exercise would be consistent with the temporal association between bone resorption and formation in a normal bone remodelling cycle. It is possible that an increase in bone formation might have been observed had the follow-up period been extended beyond 4 days as several studies report an increase in P1CP between 24 h and 96 h after exercise (Virtanen *et al.*, 1993; Thorsen *et al.*, 1997; Langberg *et al.*, 1999; Langberg *et al.*, 2000).

An increase in bone resorption, without a concomitant increase in formation, reflects an alteration to the bone remodelling balance. This has been observed previously (Guillemant *et al.*, 2004; Kerschan-Schindl *et al.*, 2009) and might, over time, result in a net loss of bone tissue (Garnero *et al.*, 1996). It is possible that if acute, exhaustive exercise is repeated frequently, such as during physical training, the transient elevation in osteoclastic activity shown in this study might explain why studies report low BMD in some endurance athletes (Bilanin *et al.*, 1989; Hind *et al.*, 2006). It has been proposed that bone resorption may play an important role in SFx development, where bone resorption transiently weakens bone, making it vulnerable to further mechanical stresses, which leads to the development of microdamage and subsequent fracture (Schaffler *et al.*, 1990). The high incidence of SFx reported in endurance runners (Johnson *et al.*, 1994; Bennell *et al.*, 1996) and military recruits (Lappe *et al.*, 2008) might, in part, result from increases in osteoclastic activity associated with repeated bouts of strenuous, acute running.

This study is the first to examine the influence of training status on the response of specific bone resorption and formation markers to acute, exhaustive exercise. There was no significant difference in basal indicators of bone metabolism between ET, RA and CON suggesting that the remodelling balance between resorption and formation remains unchanged by endurance training. Previous studies have reported higher concentrations of OC (Nishiyama *et al.*, 1988) and bone turnover markers (Hetland *et al.*, 1993) in trained individuals. However, Brahm *et al.* (1997c) reported a lower rate of bone turnover in those involved in regular physical activity compared with their less active counterparts. The different findings are probably due to the measurement of different bone turnover markers, including several less-specific markers such as total ALP, and the different subject characteristics of the non-trained groups.

This study shows no significant differences in the pattern of change in bone resorption and bone formation markers between the RA and ET groups on the four days following exhaustive running. Although the peak β-CTX concentration occurred 24 h earlier in RA, the magnitude of the increase was similar to that in ET. In addition, AUC_I analysis showed no difference in the overall effect of exercise on β-CTX, suggesting that training status does not influence the response of bone turnover to exhaustive running. Nishiyama *et al.* (1988) examined the effects of training status on changes in OC, PTH and total ALP concentrations with acute exercise. At baseline, OC concentrations were approximately twice as high in athletic (volleyball players) compared with non-athletic students, a difference which was maintained at 60 min post-exercise, although there was a more rapid increase in OC in response to exercise in the non-athletic group. Herrmann *et al.* (2007) studied β-CTX and P1NP in male athletes and sedentary controls performing 60 min of cycling at 75%, 95% and 110% of AT, although, no statistical comparison between the two groups was performed and the authors did not standardise the time of exercise or food intake. A visual inspection of their data, however, suggests no consistent difference between athletes and controls.

An examination of the literature suggests that this is the first study to examine the acute time course of changes in circulating OPG during exercise and recovery, and findings from it indicate that OPG concentrations are significantly increased after only 20 min of running in both RA and ET subjects and remain increased up to the morning following exercise. Basal OPG concentrations increase in the late morning (Joseph *et al.*, 2007) and, as this study did not measure OPG in a rested control group, at least some of the increase in OPG during exercise might have been due to its circadian rhythm. However, as the magnitude (up to 40%) of the increase with exercise was greater than that reported by Joseph *et al.*, (2007) over the same period, and greater than the CV of the assay, this suggests that much of the increase in OPG is a genuine response to exercise. Previous studies have shown increased OPG concentrations immediately following a 246 km run (Kerschan-Schindl *et al.*, 2009), a marathon (Ziegler *et al.*, 2005) and acute, resistance exercise (Brooke-Wavell *et al.*, 2007), but not following a 15.8 km run (Ziegler *et al.*, 2005). In the present study, OPG remained increased for 24 h

compared to 3 days following a 246 km run (Kerschan-Schindl *et al.*, 2009) which might reflect differences in the exercise stimuli of the two studies. Like Herrmann and Herrmann (2004), the present results indicate no effect of training status on basal OPG concentrations, which is consistent with there being no effect of 10 weeks of endurance training on basal OPG (Østergard *et al.*, 2006).

In conditions associated with significant bone loss, increased OPG concentrations have been interpreted to indicate a compensatory, albeit partially ineffective, response to increased bone resorption (Yano *et al.*, 1999; Misra *et al.*, 2003). Similarly to previous work (Kerschan-Schindl *et al.*, 2009), in the present study, both β-CTX and OPG were increased and this may reflect a similar compensatory effect. Although the temporal association of changes in β-CTX and OPG with exercise are not known, as OPG remained increased only up to 1 day post-exercise, it is possible that the initial up-regulation of OPG release matched that of RANKL, but subsequently diminished, allowing excess RANKL to stimulate osteoclasts. Serum RANKL has previously been shown to be decreased immediately after a marathon (Ziegler *et al.*, 2005) and increased at 3 days following a 246 km run (Kerschan-Schindl *et al.*, 2009). RANKL was not measured in this study as previous studies using the Biomedica RANKL assay report RANKL being undetectable in 55% of samples from postmenopausal women (Mezquita-Raya *et al.*, 2005) and 70% of healthy controls (Hegedus *et al.*, 2002). It also remains to be clearly established whether circulating OPG accurately reflects local OPG production in bone, as OPG is also expressed in the kidney, lung and heart (Simonet *et al.*, 1997).

The current study supports previous findings of an increase in circulating PTH concentrations in response to both short (≤ 1 h) (Salvesen *et al.*, 1994; Thorsen *et al.*, 1997; Zerath *et al.*, 1997; Bouassida *et al.*, 2003; Guillemant *et al.*, 2004) and longer (2 h) endurance exercise (Barry and Kohrt, 2007). PTH concentrations exhibit a distinct circadian rhythm in healthy individuals, with low concentrations during the day and a nocturnal increase that peaks in the early morning (Ahmad *et al.*, 2003; Fuleihan *et al.*, 1997). Without a non-exercising control group the possibility that some of the change in PTH seen in the present study is due to its circadian variation cannot be ruled out. However, exercise was performed in the morning, when PTH concentrations are declining, so it is likely that not only is the increase in PTH a true effect of exercise, but it is also possible that the magnitude of the increase may have been partly attenuated by basal variation.

Although some studies report PTH to be increased for 24 h or more after exercise (Brahm *et al.*, 1996; Brahm *et al.*, 1997a; Bouissida *et al.*, 2003), the present results agree with other studies that show a transient effect of exercise on PTH, with concentrations returning to baseline values within 30 min of the termination of exercise (Rudberg *et al.*, 2000; Guillemant *et al.*, 2004; Tosun *et al.*, 2006). The rapid decline in concentrations following exercise is consistent with the short (15-30 min) half life of PTH in humans (Kent *et al.*, 1985; Lindsay *et al.*, 1993).

The significant decrease in PTH concentrations from pre-exercise levels seen between 1 and 2 h post-exercise does not appear to have been previously documented in the literature. Basal PTH concentrations decline in the late morning so it is possible that the reduction in PTH seen in the present study from BASE to 1-2 h post exercise (approximately 0830 h to 0900 h through to 1200 h to 1400 h) is, in part, due to the circadian rhythm. However, the magnitude of this decline (up to 30%) appears greater than would be expected from a change due to the circadian rhythm over the same period (Fuleihan *et al.*, 1997; Ahmad *et al.*, 2003). In addition, according to the data of Fuleihan *et al.* (1997), PTH concentrations reach a nadir at around 1100 h and begin to rise thereafter. Taken together, these findings suggest that the significant reductions in PTH concentrations in the early post-exercise period are, at least in part, associated with prior exercise.

As in the present study, Nishiyama *et al.* (1988) showed no effect of training status on changes in PTH with acute exercise in young men, although others have shown that the PTH response in older men is enhanced following a physical training programme (Zerath *et al.*, 1997). These differences may be due to the different age of the subjects used and that subjects in the study of Zerath *et al.* (1997) were sedentary prior to undertaking 6 weeks of training whereas, in the present study, the RA group were all physically active and the ET group had all been running regularly for at least two years.

When given exogenously, PTH has been shown to have dual effects on human bone, with prolonged infusions increasing bone resorption and bone loss, and daily injections, which produce transient spikes in PTH concentrations, inducing bone formation (Qin *et al.*, 2004). In the present study, the effect of exercise on PTH was transient with concentrations returning to baseline within 30 min of the cessation of exercise. The rapid return of PTH concentrations to baseline levels is an important feature of its anabolic effect on bone (Frolik *et al.*, 2003) suggest the increase in PTH with running might also stimulate bone formation. However, despite the marked and transient increase in PTH, the effect of exhaustive running in the present study was to increase bone resorption only, with no change in bone formation although again, this interpretation may be limited by the duration of the follow-up period.

Although PTH is a key regulator of bone resorption, changes in glycemic status are known to alter bone resorption marker levels. β-CTX is suppressed by 35% after 45 min of steady-state hypoglycaemic (2.5 $mmol \cdot L^{-1}$) hyperinsulinemia but is unaffected by euglycaemic hyperinsulinemia (Clowes *et al.*, 2002b). In the present study, modest (3.5 $mmol \cdot L^{-1}$) hypoglycaemia was evident at the end of exercise and for up to 2 h post-exercise. Hypoglycaemia with endurance running is unusual, occurring more frequently towards the end of high intensity, long duration cycling. In addition, glucose levels do not typically fall as low as those during insulin-induced hypoglycaemia studies (Shearing *et al.*, 1992; Clowes *et al.*, 2002b). Interestingly, data from Guillemant *et al.* (2004) shows a significant increase in β-CTX with 60 min of 80% VO_{2max} that begins only in the later part of exercise and is maximally expressed in the first 2 h post-exercise. Blood glucose was not measured,

but similar exercise does result in a significant reduction in blood glucose (Ronsen *et al.*, 2002). Although Clowes *et al.* (2002b) showed hypoglycaemia reduced rather than increased β-CTX concentrations, this was with hypoglycaemic hyperinsulinemia rather than the hypoglycaemic hypoinsulinemia, which existed after exercise in the present study. It is possible, although speculative, that hypoglycaemic hypoinsulinemia has a different effect on bone resorption although how this could be investigated in an *in vivo*, non-exercising model in humans is unclear.

Several mechanisms by which exercise results in an increase in PTH have been proposed including a lowering of systemic calcium concentrations, metabolic acidosis, adrenergic stimulation and an increase in PO_4. The increases in calcium with exercise in the present study agree with a number of previous studies (Ljunghall *et al.*, 1986; Salvesen *et al.*, 1994; Brahm *et al.*, 1997a; Tsai *et al.*, 1997; Guillemant *et al.*, 2004) and only one study reports a drop in total Ca associated with an increase in PTH with acute exercise (Nishiyama *et al.*, 1988). Barry and Kohrt (2007) report increases in both total Ca and PTH immediately after 2 h of cycling but, when concentrations were corrected for changes in plasma volume, total Ca was decreased. Although this suggests that the decrease in serum Ca may have triggered the increase in PTH, the decrease in total calcium did not correlate with the increase in PTH. Also, importantly, as the secretion of PTH by the parathyroid glands is determined by the calcium-sensing receptor's detection of changes in the iCa concentration (Tfelt-Hansen and Brown, 2005), adjusting calcium concentrations for changes in plasma volume may not be appropriate.

Ionised calcium was not measured in this study and several previous studies report increases in PTH concomitant with decreases in iCa (Nishiyama *et al.*, 1988; Thorsen *et al.*, 1997; Bouissida *et al.*, 2003; Maïmoun *et al.*, 2005; Maïmoun *et al.*, 2009). However, some studies also report increases in iCa with increased PTH (Henderson *et al.*, 1989; Ljunghall *et al.*, 1984b) while others report increases in iCa without a decrease in PTH (Ljunghall *et al.*, 1984b; Cunningham *et al.*, 1985; Ljunghall *et al.*, 1985; Ashizawa *et al.*, 1997; Thorsen *et al.*, 1997; Rong *et al.*, 1997; Rudberg *et al.*, 2000). Taken together, the results of previous studies suggest that the feedback loop between iCa and PTH that is evident in resting individuals does not explain the increase in PTH with acute exercise in the present study.

The increase in PTH may, however, actually have contributed to the increase in ACa, in the short term by stimulating calcium reabsorption in the kidney, and then by osteoclastic resorption of bone, releasing additional calcium into the circulation (Fraser, 2009). This would explain the peak in calcium concentrations occurring at the end of IEE, at which point the duration of exposure to increased PTH was greatest. Unlike Nishiyama *et al.* (1988), the present results suggest that the increase in calcium induced by running is enhanced with improved training status. This enhancement might be explained by the ET group exercising for approximately 50% longer than RA, resulting in a longer exposure to increased PTH, increased bone resorption and calcium liberation. It is also

possible that the increased calcium concentrations in the 2 h post-exercise contributed to the significant decrease in PTH during the same period.

Although blood pH wasn't measured in this study, given the modest elevations in blood lactate, and the increased PTH during moderate intensity (70 to 75% of VT) endurance exercise (Barry and Kohrt, 2007), metabolic acidosis would also appear an unlikely mechanism for the effect of exercise on PTH. Additionally, endurance exercise at a higher (80% VO_{2max}) intensity than that used in the present study is associated with higher lactate concentrations (>4 $mmol \cdot L^{-1}$) but no decrease in blood pH (Peinado *et al.*, 2006) further supporting this idea.

The moderate hypoglycaemia seen at the end of exhaustive running is unlikely to have been involved in the increase in PTH as PTH concentrations decrease rather than increase during hypoglycaemia (Shearing *et al.*, 1992; Clowes *et al.*, 2002b) and PTH was increased through the early part of exercise when blood glucose concentrations were also increased. The modest hypoglycaemia seen at the end of exercise, however, might provide an alternative explanation as to why PTH concentrations were reduced slightly from those in FD, despite the higher exercise intensity.

Considerable evidence indicates that adrenergic agonists can increase PTH (Heath, 1980) although further studies are required to determine whether this effect is evident with physiological increases in catecholamines (Body *et al.*, 1983; Vora *et al.*, 1983). Any increase in catecholamines in the present study is likely to have been modest, as marked increases are reported to occur only at exercise intensities close to the lactate threshold (Mazzeo and Marshall, 1989; Weltman *et al.*, 1994) and lactate concentrations showed only modest increases in both the RA and ET groups.

PO_4 concentrations were increased promptly with exercise and remained elevated at 2 h post-exercise. As with PTH, PO_4 has a pronounced circadian rhythm with declining concentrations during the morning, so it is likely that the increase in PO_4 is indeed a true effect of exercise. In circadian rhythm studies, the correlation between the change in PTH and that in PO_4 is strongest when PTH lags PO_4 (Ahmad *et al.*, 2003), suggesting a physiological relationship between PO_4 and PTH. Relatively small (<50%) increases, in the circulating PO_4 concentration are associated with increases in PTH concentrations (Silverberg *et al.*, 1986; Kärkkäinen and Lamberg-Allardt, 1996; Martin *et al.*, 2005) and previous studies of endurance exercise report both increased PTH and PO_4 (Ljunghall *et al.*, 1986; Guillemant *et al.*, 2004; Maïmoun *et al.*, 2006). Although some studies suggest that the effect of PO_4 on PTH is relatively slow, with PTH not increased until 45 to 60 min after PO_4 ingestion (Estepa *et al.*, 1999), a more recent study in rats reports increases in both PO_4 and PTH after only 10 min of a sodium phosphate infusion (Martin *et al.*, 2005). As PO_4 was increased 25-30% after only 20 min of exercise in the present study, this suggests that the increase in PO_4 might have resulted in an increase in PTH. That said, however, there was no significant relationship between changes in PO_4 and in PTH during exercise. As changes in PTH lag those in PO_4 (Fraser *et al.*, 1998; Ahmad *et al.*, 2003), the lack of a

relationship does not preclude a stimulatory effect of PO_4 on PTH, as both PTH and PO_4 were increased at EX20. Further studies are required that make more frequent measures in the early part of exercise to explore this relationship.

As in previous studies (Guillemant *et al.*, 2004; Maïmoun *et al.*, 2006), PO_4 concentrations decreased in the early post-exercise period but remained elevated from baseline. Like ACa, the sustained increase in PO_4 in the first 2 h post-exercise might be attributed to the liberation of PO_4 from bone by PTH-mediated osteoclastic bone resorption. The marked decrease in insulin concentrations, reducing the insulin-stimulated uptake of PO_4 by soft-tissues might also have contributed to this increase (Soskin *et al.*, 1941). Taken together, the present results might suggest that increased PTH with exercise increases bone resorption, reflected by increased β-CTX levels, which in turn increases Ca and PO_4 and induces a compensatory increase in OPG.

This study has several important limitations. Firstly, the RA and ET were defined in terms of their history of endurance activity and on cardiorespiratory parameters (*e.g.* VO_{2max}). Although the ET group had a significantly higher quantity of weekly training and significantly higher VO_{2max}, the RA group did have had a history of weight-bearing exercise. As such, it is likely that the bones of their skeletons were accustomed (in a generalised way) to the forces applied to them during treadmill running. As the responsiveness of bone to mechanical loads appears to diminish as the duration of a loading programme is extended (Kim *et al.*, 2003; Saxon *et al.*, 2005; Schriefer *et al.*, 2005), this might account for the lack of any significant differences in changes in bone turnover markers between the RA and ET groups and a more marked response have might be observed in a population who were truly naive to the exercise protocol. However, testing a truly naive population would require a carefully designed study, as even the completion of a VO_{2max} test for the purposes of exercise prescription would render subjects no longer naive to running. Naive subjects might also have considerable difficulty in completing an exercise protocol similar to the one used in this study.

Secondly, although all subjects exercised to the same relative end point (*i.e.* volitional fatigue), total exercise duration varied considerably within both groups and mean duration tended to be greater in the ET group. This would have resulted in a wide individual variation in the total number of mechanical loads and, on average, a greater number in the ET group. Additionally, body mass varied from 59.6 kg to 96.5 kg across the RA and ET groups and mean body mass was significantly lower in the ET group, while running speed was significantly higher. A lower body mass would result in lower GRFs during running and might result in lower strain magnitudes and strain rates in bone.

In contrast, the higher mean running speed in the ET group will likely result in higher strains and strain rates in bone (Burr *et al.*, 1996). However, this relationship is complicated by the finding that ground reaction forces during running are higher in non-active subjects than in runners (Karamanidis and Arampatzis, 2005), while sedentary individuals also present with similar peak accelerations than

runners despite a lower running speed (Dériaz *et al.*, 2010). In addition, it is also likely that the skeletons of more aerobically fit runners are adapted to the mechanical loads imposed by the higher running speeds. Therefore, faster running speeds will not necessarily result in larger strain magnitudes and strain rates. Thus, variability in exercise duration, body mass and running speed might all have contributed to the findings from this study.

In conclusion, there was no effect of training status on the bone turnover response to acute, exhaustive running. However, there was a marked and sustained increase in bone resorption in both groups in response to exercise, which was not accompanied by an increase in bone formation in the short-term (4 days). This suggests a different effect of exhaustive exercise on the bone turnover process during recovery, which might explain previous findings of deleterious effects on the skeleton in those who partake in regular strenuous, endurance exercise. The increase in serum OPG concentrations might represent a compensatory, albeit ineffective, response to the increase in bone resorption or could simply be a direct effect of exercise. The increase in PTH may be responsible for the increase in bone resorption but, unlike at rest, this increase cannot be explained by decreased calcium but might, in part, be related to the increase in PO_4.

CHAPTER V

STUDY II – THE EFFECT OF RECOVERY DURATION ON THE METABOLIC RESPONSE OF BONE TO TWO BOUTS OF ACUTE RUNNING

5.1 Introduction

In Study I, compared to non-exercising controls, acute exhaustive running was associated with an increase in β-CTX, but not P1NP or bone ALP for four days following exercise. An increase in bone resorption without a concomitant increase in bone formation indicates an alteration to the bone remodelling balance, favouring bone resorption. As both athletes and military recruits perform training sessions on consecutive days the results from the previous study suggests that following a particularly strenuous bout of exercise, subsequent bouts might be performed at a time when bone resorption is elevated.

Despite the obvious importance of the repetitive application of mechanical loads in changes in bone quality and structure, there has been relatively little research into the effects of short periods of repeated exercise on BTM. Several studies have examined short periods of training (Fujimura *et al.*, 1997; Woigte *et al.*, 1998a; Langberg *et al.*, 2001; Adami *et al.*, 2008; Evans *et al.*, 2008; Lester *et al.*, 2009) and indicate that training can result in changes in bone turnover. None of these studies, however, have examined changes in BTM over a period of training of less than 3 weeks.

Several studies have examined changes in BTM over much shorter periods of repeated exercise (Zanker and Swaine, 2000; Ihle and Loucks, 2004). These studies showed that when a bout of acute, weight bearing exercise was repeated daily for between three and five days, in volunteers who were in energy balance, there was no significant change in bone turnover markers. However, neither of these studies measured β-CTX and only the study of Zanker and Swaine, (2000) measured P1NP. These studies only measured BTM before and after the period of training and no studies have examined the acute time course of changes in BTM in response to multiple bouts of exercise.

In addition to performing training sessions on consecutive days, athletes and recruits may perform more than one training session on the same day. Recently, it has been shown that a second bout of exercise on the same day induces more pronounced changes in leukocyte subsets, stress hormones including epinephrine and growth hormone, and the cytokines IL-6 and IL-1ra compared with a single bout of identical exercise (Ronsen *et al.*, 2000; Ronsen *et al.*, 2001; Ronsen *et al.*, 2002). In some instances, these increases occur despite concentrations returning to baseline levels prior to the second bout. In Study I, PTH increased promptly with exercise and remained increased throughout and may have mediated the increase in bone resorption. Although PTH returned to baseline rapidly with the

termination of exercise, based on the results above, it is possible that the PTH response to exercise might be altered during a second bout of exercise performed on the same day. When Bouassida *et al.* (2003) compared 42 min of continuous exercise with two, 21 min bouts separated by 40 min, they observed significantly lower, rather than higher, PTH concentrations immediately, and at 24 h after the second bout compared with continuous exercise. No studies have examined the effect of recovery duration between repeated bouts of exercise on changes in bone metabolism.

As reduced spinal BMD levels have been reported in some endurance athletes, and both athletes and military recruits suffer a high incidence of SFx, it is of interest to understand how repeated bouts of exercise and the recovery duration between consecutive bouts influences bone metabolism. These findings may help guide the scheduling of activity and recovery during periods of repeated exercise to minimise unfavourable changes in bone turnover.

The purpose of this study was to examine the effect of two bouts of acute, weight-bearing exercise performed on consecutive days on bone metabolism. Additionally, using an ecologically valid model of exercise, recovery and nutrition, this study also investigated the response of bone metabolism when recovery duration between the two bouts of exercise was reduced from 23 h to 3 h.

5.2 Materials and Methods

5.2.1 Subjects

Ten physically-active men were recruited to participate in the study (Table 5.1). All subjects were in good physical condition, with a history of weight-bearing exercise and average to above average levels of fitness. The group was a mixture of team sports players and recreational runners and all subjects performed at least bout of endurance running per week. The study had a within-subject design with all subjects completing all experimental conditions.

5.2.2 Overview of exercise intervention

On Days 4 and 5, all subjects performed two, 60 min bouts of treadmill running (exercise bouts A [ExA] and B [ExB]) separated by a recovery period of either 23 h (LONG) or 3 h (SHORT). In LONG, exercise was performed at 1415 h on Day 4 (L-ExA) and an identical bout at 1415 h on Day 5 (L-ExB). In SHORT, subjects rested on Day 4 and completed both bouts of running exercise on Day 5 at 1015 h (S-ExA) and 1415 h (S-ExB) (Figure 5.1). The order in which subjects completed the two conditions was counterbalanced.

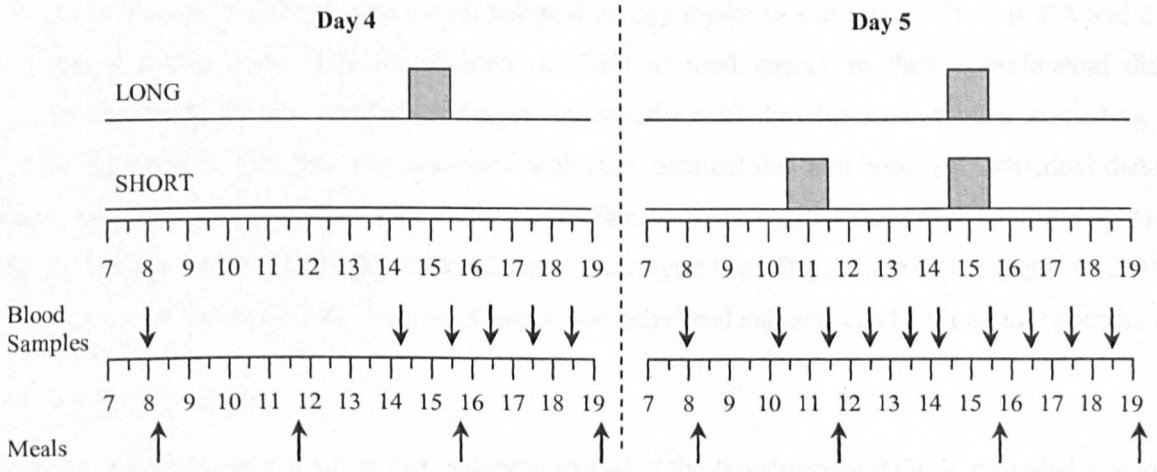

Figure 5.1. Diagram showing the exercise protocol on Days 4 and 5 in the SHORT (top) and LONG (bottom) experimental conditions. Grey boxes indicate exercise bouts A and B (60 min at 65% VO_{2max}). Vertical arrows indicate timings of blood samples and standardised meals.

5.2.3 Preliminary Measures

Medical Screening: Subjects underwent a full medical examination as described in Section 3.2.1.

Dietary Analysis: Subjects completed a three-day food diary as described in Section 3.2.2, which was subsequently analysed as described in Section 3.2.2.

Determination of the association between oxygen uptake and running velocity during level (0° gradients) treadmill running and of maximal rate of oxygen uptake: The association between O_2 uptake and running velocity during level running was determined as described in Section 3.2.3. VO_{2max} was determined as described in Section 3.2.4. The results of the two tests were used to estimate the treadmill velocity corresponding to 50% and 65% VO_{2max} during level running based on the regression line of VO_2 and treadmill velocity.

Experimental Dietary Provision: Measurements of skin fold thickness using calibrated callipers were taken in triplicate at four sites (bicep, tricep, subscapular and suprailiac), and estimates of fat-free mass, fat mass, and percentage body fat were subsequently calculated using the formulas of Durnin and Womersley, (1974). In Study I, designing diets that contained 8 g CHO·kg FFM^{-1} but remained isocaloric with subjects' habitual diet, resulted in an experimental diet with a high CHO content, particularly if subjects did not have a high habitual energy intake (3 subjects in ET, 5 in RA and 2 in CON had a greater than 70% contribution of CHO to total energy in their experimental diet). Thus, in this study it was decided to design an experimental diet for each subject consisting of 6 g CHO·kg FFM^{-1}. This diet was isocaloric with their habitual diet and based on individual dietary habits. Subjects were provided with three menus that were given in a three-day cyclic order with menu A on Days 1 and 6, menu B on Days 2 and 7, and menu C on Days 3 and 8 (for details of diet on Days 4 and 5 see *Section 5.2.4*). Details of menus for individual subjects can be found in Appendix A.

5.2.4 Trial Procedures

On Day 4, following an overnight fast, subjects arrived at the laboratory at 0730 h, provided a second void urine sample and had their body mass measured (Mettler-Toledo ID7, Mettler-Toledo, Germany). Subsequently, subjects adopted a semi-recumbent position on a bed and a blood sample was collected by venepuncture at 0800 h. At 1400 h a cannula (18GA 1.2x45 mm, Becton Dickinson, USA) was inserted into a vein in the forearm, where it remained until after the final blood sample. On Day 5, following an overnight fast, subjects arrived at the laboratory at 0730 h, provided a second void urine sample, had their body mass measured and adopted a semi-recumbent position on a bed. A cannula was inserted into a vein in the forearm where it remained until 1830 h.

Exercise bouts were equal in intensity and duration and consisted of a 5 min warm-up at 50% VO_{2max} followed by 60 min at 65% VO_{2max}, separated by 5 min for volitional stretching. All subjects completed all exercise bouts, but the running speed of one subject had to be reduced temporarily

during ExB in SHORT to avoid premature exhaustion. The subject who had his running speed reduced was excluded from the data analysis of cardiorespiratory variables during exercise.

Samples of expired air were collected for 60 sec after 14, 29, 44 and 59 min of exercise and were analysed as described in Section 3.3.1. Prior to, and following both bouts of exercise, subjects had their body mass measured and any loss in body mass was replaced with plain water.

Blood samples were collected at identical time points in both conditions; on Day 4 at 0800 h, 1415 h, 1530 h, 1630 h, 1730 h, and 1830 h; and on Day 5 at 0800 h, 1015 h, 1130 h, 1230 h, 1330 h, 1415 h, 1530 h, 1630 h, 1730 h, and 1830 h (Figure 5.1).

On both days of each condition, subjects consumed a standardised diet (13.3 MJ, 52% CHO, 33% FAT, 15 % PRO) divided into four meals. This diet was calculated from a standard diet of 2500 kcal plus a supplement on Days 4 and 5 to account for the increased energy expenditure associated with each bout of exercise. As mean running speed in Study I in the RA group at 65% VO_{2max} was 10.0 $km \cdot h^{-1}$, the supplement was chosen to be 660 kcal, as Åstrand and Rodahl (1986), report an energy expenditure of approximately 11 $kcal \cdot min^{-1}$ with running at 9 $km \cdot h^{-1}$ and this was the closest energy expenditure data available. The first three meals were eaten in the laboratory at 0815 h, 1145 h, and 1545 h, and subjects took the fourth meal home to be consumed between 1900 h and 2100 h (Figure 5.1). The first three meals consisted of sandwiches and cereal bars; one sandwich and one cereal bar were served at 0800 h and 1145 h, and two of each at 1545 h. Subjects were given a pre-packaged meal and two cereal bars for their evening meal.

The ambient room temperature was 23.1 ± 1.1°C and 21.4 ± 1.3°C in the metabolic and exercise laboratory respectively.

5.2.5 Biochemical analysis

All blood samples drawn on Days 4 and 5 were analysed for glucose, lactate, OPG, PTH, ACa and PO_4. Samples drawn at 0800 h were also analysed for β-CTX, P1NP, bone ALP. Samples on the four follow-up days (FU1 – FU4) were analysed for β-CTX, P1NP, bone ALP, OPG, PTH, ACa and PO_4. All urine samples were analysed for fPYD, fDPD and Cr and the volume of each sample was also recorded. Pyridinoline concentrations were expressed as a ratio with Cr concentrations. Pyridinoline 'output' (in nmol) was also calculated by multiplying pyridinoline concentrations ($nmol \cdot L^{-1}$) by urine volume (L).

5.2.6 Statistical Analysis

All data are presented as mean ± 1SD unless otherwise stated. Statistical significance was accepted at an alpha level of $P < 0.05$. Paired samples t-tests were used to compare habitual and experimental dietary data and baseline biochemistry. A one-way ANOVA was used to compare the change in glucose, lactate, OPG, PTH, ACa and PO_4 from pre- to immediately post-exercise in the four exercise bouts.

All biochemical data, body mass and variables relating to exercise were analysed using a LMM, with the factors *Time* (of sampling) and *Condition* (LONG *vs* SHORT) included and with individuals as a random within-group factor. The assumptions of the LMM were investigated by examining the distribution of residuals and the pattern of residuals versus fitted values. Where non-normality or non-constant variance was observed, a transformation was applied to the data so that the assumptions were satisfied. Body mass, β-CTX, P1NP, bone ALP and albumin-adjusted calcium did not require transformation. Normality and homogeneity were achieved following log transformations for all other variables. For bone turnover markers, Cr and urine volume, data was analysed from measurements taken at BASE, 0800 h on Day 5 (D5) and the four follow-up days (FU1 – FU4). In order to examine the effect of recovery duration on responses to a second bout of exercise, data from glucose and lactate measurements from 1415 h through to 1830 h on Day 5 were included, and data from measurements of OPG, PTH, ACa and PO_4 were included from 1415 h on Day 5 through to FU4.

Where there was a significant main effect of *Time* but no significant *Condition* x *Time* interaction, each subsequent time point was compared against BASE using a pooled mean using Dunnett's test with BASE as the 'Control'. When the *Condition* x *Time* interaction was significant, within each group, each subsequent time point was compared against BASE using Dunnett's test with BASE as the 'Control' and groups were compared to each other at all time points using the SNK test.

For all four exercise bouts, Pearson's correlation coefficient was calculated between the percentage changes in PO_4 and PTH from pre-exercise to immediately post-exercise.

5.3 Results

5.3.1 Subject characteristics

Subject characteristics are shown in Table 5.1.

Table 5.1. Subject characteristics.

Measure	Value
n	10
Age (y)	26.5 ± 5.1
Body Mass (kg)	78.3 ± 5.8
Height (m)	1.79 ± 0.05
BMI ($kg \cdot m^{-2}$)	24.5 ± 1.9
FFM (kg)	67.8 ± 4.8
Body Fat (%)	14.3 ± 4.3
VO_{2max} ($ml \cdot min^{-1} \cdot kg^{-1}$)	57.3 ± 6.9

Values are mean ± 1SD. BMI, body mass index; FFM, fat-free mass.

5.3.2 Dietary analysis and experimental dietary provision

Dietary analysis: Energy intake, macronutrient composition and dietary calcium from food diary analysis are shown in Table 5.2.

Table 5.2. Habitual energy intake, macronutrient composition and dietary calcium content.

Measure	Value
Energy (MJ)	10.9 ± 2.4
CHO (g)	349.1 ± 96.7
CHO ($g \cdot kg\ FFM^{-1}$)	5.2 ± 1.5
CHO (% of total energy)	50.5 ± 3.9
FAT (% of total energy)	29.7 ± 6.6
PRO (% of total energy)	17.6 ± 2.4
Calcium ($mg \cdot day^{-1}$)	1321 (range, 803 – 2257)

Values are mean ± 1SD unless otherwise stated. CHO, carbohydrate; FFM, fat-free mass.

Experimental dietary provision: The energy content and macronutrient composition of the experimental diets are shown in Table 5.3. There was no significant difference between habitual energy intake and that provided by the experimental diet ($P = 0.287$). Experimental diets provided a smaller quantity of protein (15.6 % *vs* 17.6%, $P < 0.01$) and a greater quantity of CHO

(56.8% *vs* 50.5%, $P < 0.05$) as a percentage of total energy compared with habitual diets but there were no other differences between other habitual and experimental dietary variables.

Table 5.3. Energy content and macronutrient composition of the experimental diets consumed by subjects on Days 1-3 and 6-8 of each experimental condition.

Measure	Value
Energy (MJ)	11.4 ± 1.4
CHO (g)	400.5 ± 26.6
CHO (g·kg FFM^{-1})	6.0 ± 0.1
CHO (% of total energy)	56.8 ± 5.7 [a]
Fat (% of total energy)	27.3 ± 5.6
Protein (% of total energy)	15.6 ± 0.7 [b]
Calcium (mg·day^{-1})	1296 (range, 971 - 1652)

Values are mean ± 1SD unless otherwise stated. CHO, carbohydrate; FFM, fat-free mass. [a] different ($P < 0.05$) from habitual diet; [b] different ($P < 0.01$) from habitual diet.

5.3.3 Baseline biochemistry

There were no significant differences in baseline concentrations of any biochemical marker between the LONG and SHORT conditions (Table 5.4).

Table 5.4. Baseline biochemistry in LONG and SHORT conditions.

Variable	LONG	SHORT	P Value (paired samples t-test)
β-CTX (ug·mL^{-1})	0.59 ± 0.17	0.59 ± 0.19	0.872
P1NP (ug·mL^{-1})	56 ± 20	56 ± 17	0.975
Bone ALP (U·L^{-1})	23 ± 7	24 ± 7	0.340
Cr (umol·L^{-1})	9.5 ± 6.4	9.7 ± 6.2	0.916
PYD/Cr (nmol·mmol Cr^{-1})	16.2 ± 4.1	16.8 ± 5.2	0.543
DPD/Cr (nmol·mmol Cr^{-1})	4.1 ± 0.9	4.2 ± 1.2	0.731
OPG (pmol·L^{-1})	3.2 ± 1.1	2.9 ± 0.9	0.202
PTH (pmol·L^{-1})	3.5 ± 1.4	3.3 ± 0.9	0.521
ACa (mmol·L^{-1})	2.33 ± 0.09	2.34 ± 0.07	0.335
PO_4 (mmol·L^{-1})	1.04 ± 0.13	1.08 ± 0.15	0.647

Values are mean ± 1 SD.

5.3.4 Body mass

There was no significant main effect of *Time* (P = 0.679) and no significant *Condition* x *Time* interaction (P = 0.983) for body mass measured from Day 4 (BASE) to Day 8 (FU4) (Figure 5.2).

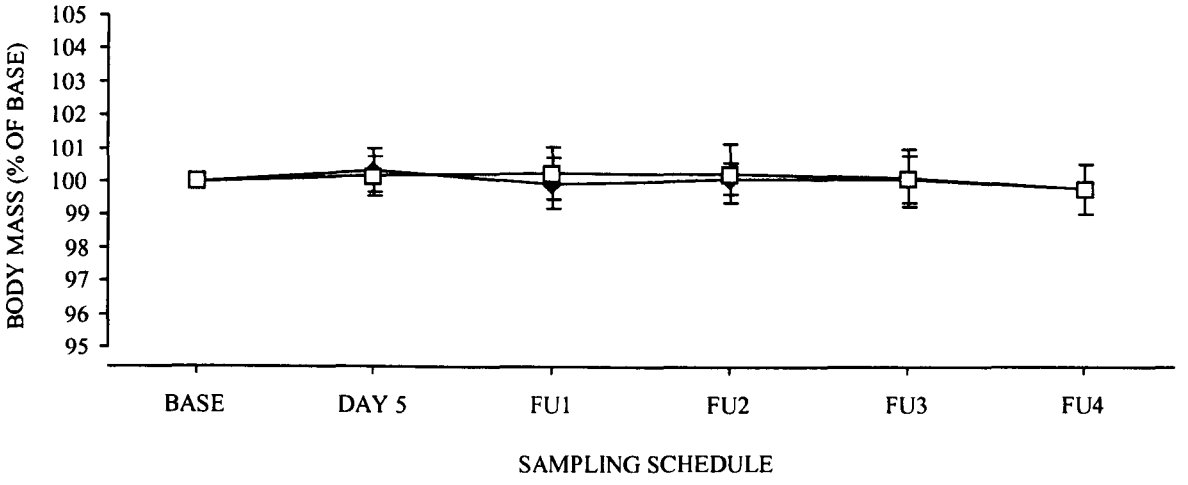

Figure 5.2 Percentage change from baseline (BASE) in body mass on Day 5 and the four follow-up days (FU1 – FU4) in the LONG (open squares) and short (filled diamonds) conditions. Values are mean ± 1SD.

5.3.5 Exercise and cardiorespiratory variables

Mean running speed was 10.1 (range, 8.7 to 12.3) $km \cdot h^{-1}$. Exercise intensities were 63.8 ± 2.8 % and 63.6 ± 2.6 % VO_{2max} in ExA and ExB in LONG and 63.9 ± 2.2 % and 65.4 ± 2.5 % VO_{2max} in ExA and ExB in SHORT. Comparing responses to ExB alone, oxygen uptake ($P < 0.01$), heart rate ($P < 0.05$), respiratory exchange ratio ($P < 0.01$) and perceived exertion ($P < 0.001$) were all significantly higher in SHORT compared with LONG (Table 5.5).

Table 5.5. Oxygen uptake (VO_2), heart rate (% of HR_{max}) respiratory exchange ratio (RER) and ratings of perceived exertion (RPE) during ExB in the LONG and SHORT conditions.

Variable	LONG	SHORT
VO_2 ($L \cdot min^{-1}$)	2.80 ± 0.28	2.91 ± 0.41 [b]
HR (% of HR_{max})	84 ± 3	87 ± 5 [a]
RER	0.891 ± 0.030	0.876 ± 0.028 [b]
RPE	12.6 ± 1.1	13.3 ± 1.4 [c]

Values are mean ±1SD. [a] different ($P < 0.05$) from LONG; [b] different ($P < 0.01$) from LONG; [c] different ($P < 0.001$) from LONG.

5.3.6 Glucose and lactate

Glucose: There was no significant differences (P = 0.507) between the changes in glucose from pre- to immediately post-exercise during the four bouts of exercise (Figure 5.3, Panel A).

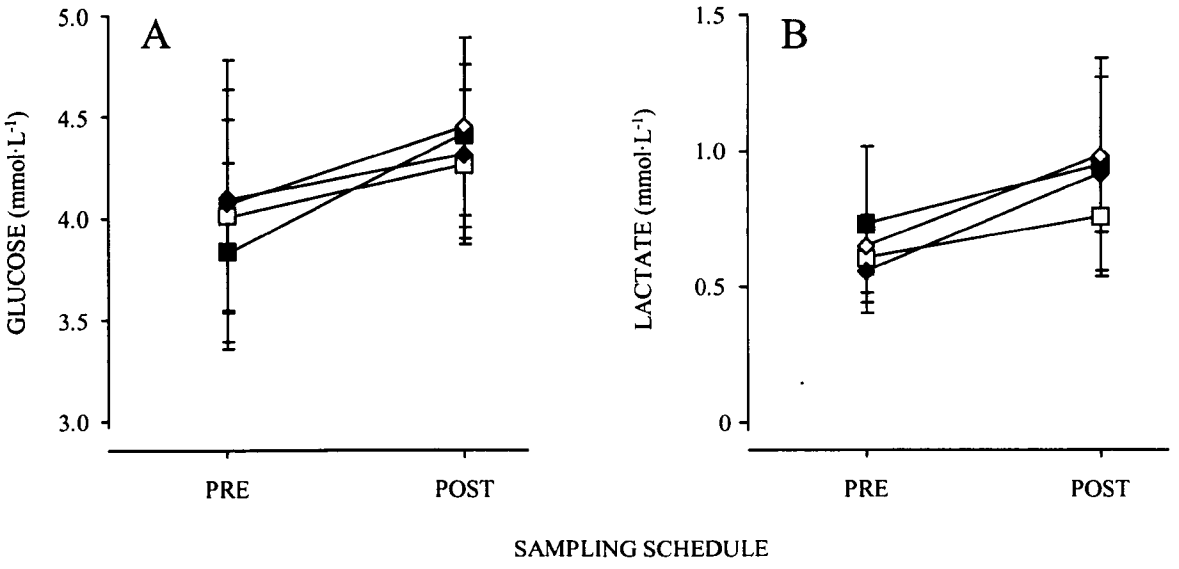

Figure 5.3 Change in glucose (Panel A) and lactate (Panel B) from pre- to immediately post-exercise in L-ExA (filled squares), L-ExB (open squares), S-ExA (open diamonds), S-ExB (filled diamonds). Values are mean ± 1SD.

When data was examined from 1415 h to 1830 h on Day 5, there was a significant main effect of *Time* ($P < 0.001$) but no significant *Condition* x *Time* interaction ($P = 0.788$) for blood glucose concentrations. Immediately post-exercise, pooled, mean concentrations were not significantly different from those measured at 1415 h (Figure 5.4, Panel A). At 1630 h, concentrations were 6.8 ± 1.2 and 6.7 ± 0.8 mmol·L^{-1} in the LONG and SHORT conditions and significantly ($P < 0.001$) increased from 1415 h. Concentrations declined thereafter but remained significantly elevated from pre-exercise at 1730 h ($P < 0.001$) and 1830 h ($P < 0.001$).

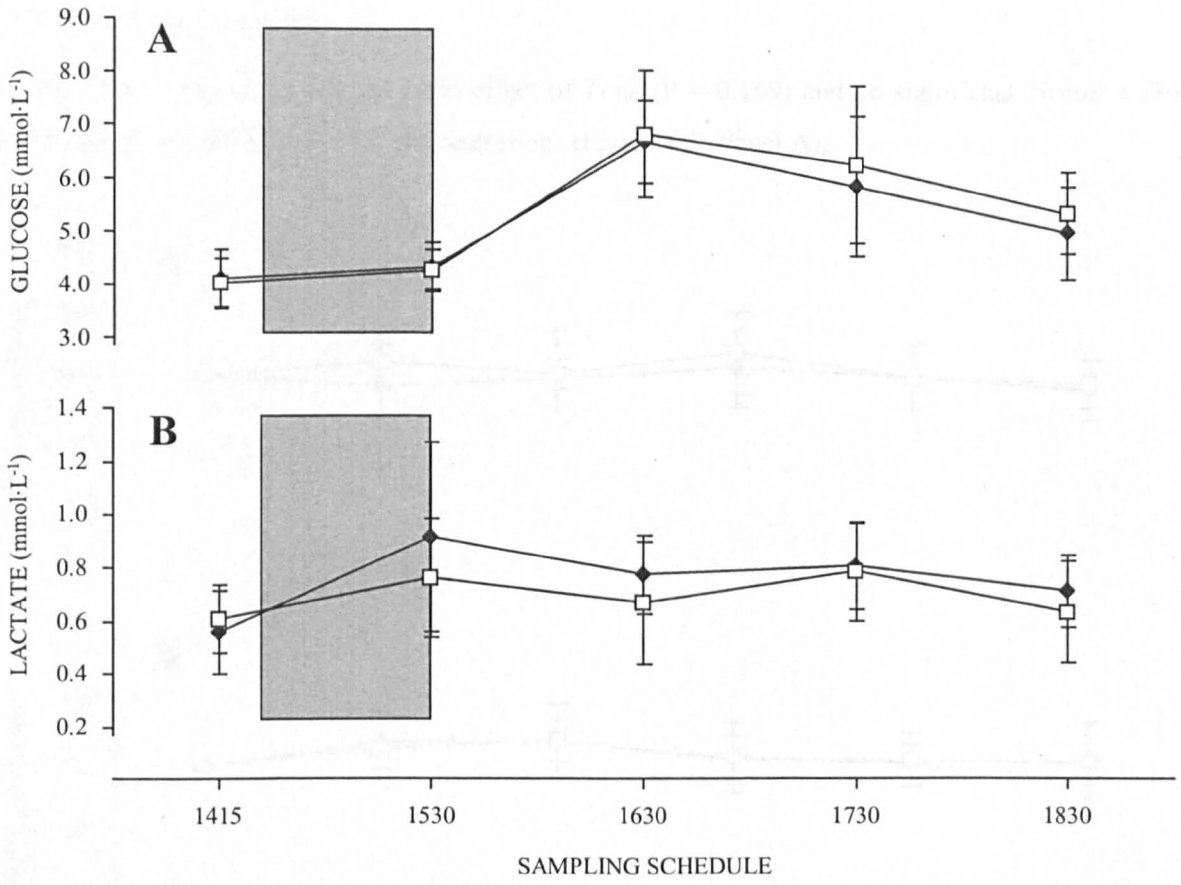

Figure 5.4. Blood glucose (Panel A) and blood lactate (Panel B) concentrations before and after exercise bout B (ExB) in the LONG (open squares) and short (filled diamonds) conditions. Grey box denotes ExB. Values are mean ± 1SD. Pooled, glucose concentrations were significantly higher than 1415 h at 1630 h, 1730 h and 1830 h. Pooled, lactate concentrations were significantly higher than 1415 h at 1530 h, 1630 h and 1730 h.

Lactate: There was no significant difference ($P = 0.326$) when comparing the absolute change in lactate from pre- to immediately post-exercise in ExA and ExB in the LONG and SHORT conditions (Figure 5.3, Panel B). When data was examined from 1415 h to 1830 h on Day 5, there was a significant main effect of *Time* ($P < 0.001$) but no significant *Group* x *Time* interaction ($P = 0.296$) for lactate concentrations. Immediately post-exercise, pooled, mean concentrations were significantly ($P < 0.001$) increased from 1415 h (0.6 ± 0.1 to 0.8 ± 0.2 and 0.6 ± 0.2 to 0.9 ± 0.4 in the LONG and SHORT conditions) (Figure 5.4, Panel B). Concentrations declined thereafter but remained significantly elevated from pre-exercise at 1630 h ($P < 0.05$) and 1730 h ($P < 0.001$).

5.3.7 Bone turnover markers

β-CTX: There was no significant main effect of *Time* (P = 0.169) and no significant *Group* x *Time* interaction (P = 0.892) for β-CTX concentrations (Figure 5.5, Panel A).

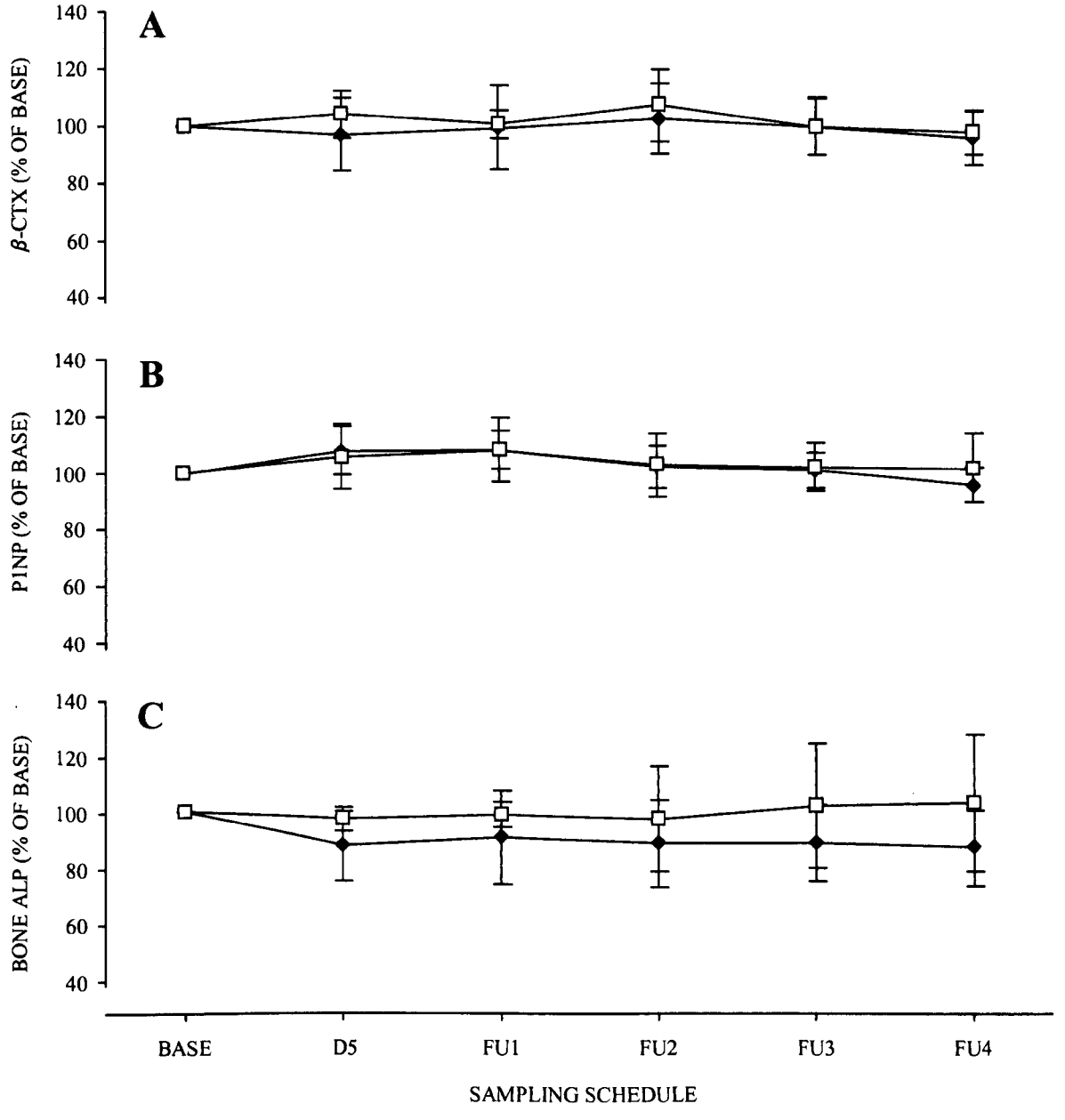

Figure 5.5. Percentage change in BASE concentrations of β-CTX (PANEL A), P1NP (PANEL B) and bone ALP (PANEL C) on Day 5 (D5) and on the four follow up days (FU1 – FU4) in the LONG (open squares) and SHORT (filled diamonds) conditions. All samples collected at 0800 h after an overnight fast. Values are mean ± 1SD.

P1NP: There was no significant main effect of *Time* (P = 0.133) and no significant *Group* x *Time* interaction (P = 0.919) for P1NP concentrations (Figure 5.5, Panel B).

Bone ALP: There was no significant main effect of *Time* (P = 0.289) and no significant *Group* x *Time* interaction (P = 0.529) for bone ALP concentrations (Figure 5.5, Panel C).

fPYD/Cr: There was no significant main effect of *Time* (P = 0.648) and no significant *Group* x *Time* interaction (P = 0.819) for fPYD/Cr concentrations (Figure 5.6, Panel A).

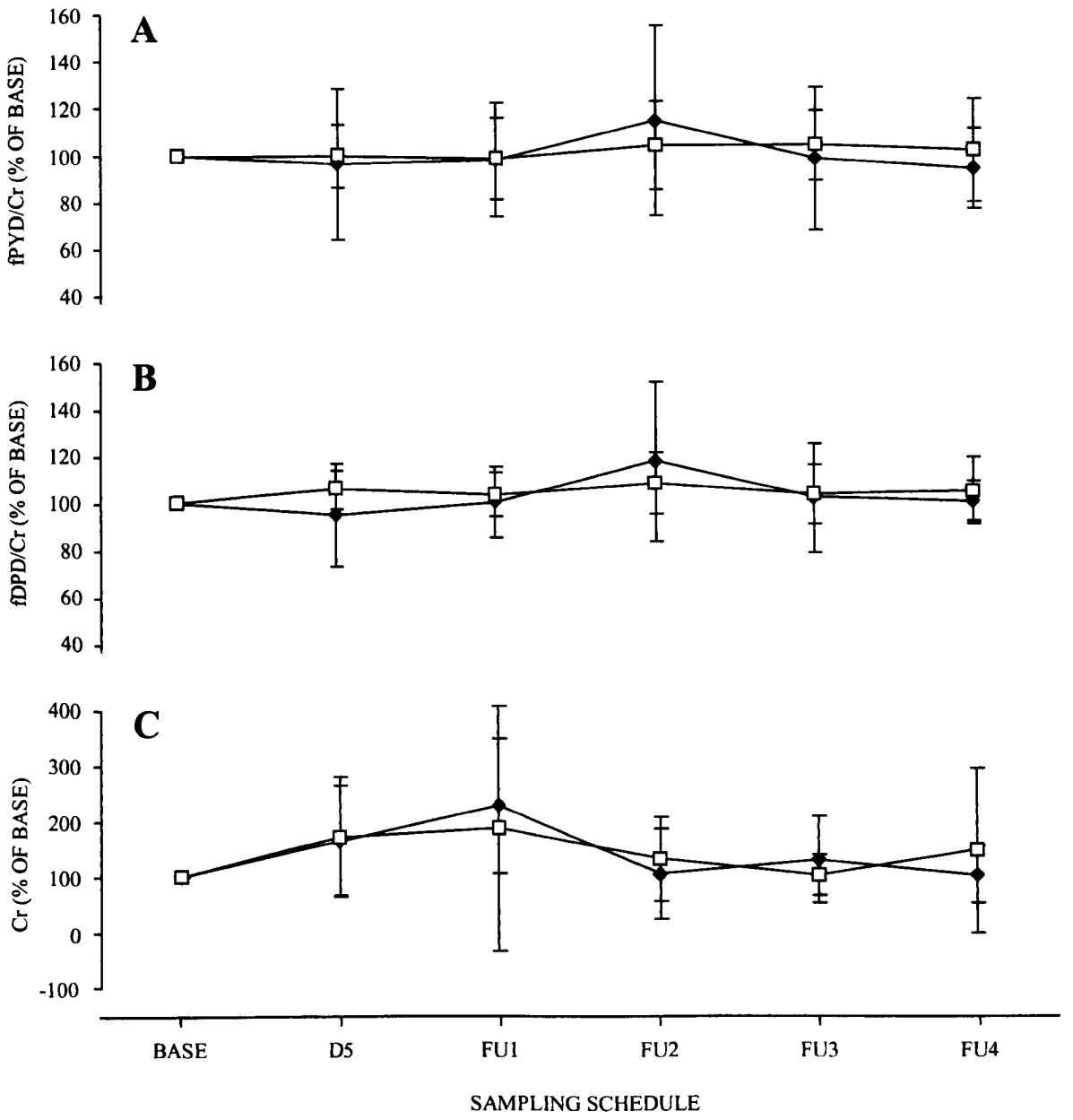

Figure 5.6. Percentage change in BASE concentrations of fPYD/Cr (Panel A) and fDPD/Cr (Panel B) and Cr (Panel C) on Day 5 (D5) and on the four follow up days (FU1 – FU4) in the LONG (open squares) and SHORT (filled diamonds) conditions. Samples collected between 0730 h and 0800 h after an overnight fast. Values are mean ± 1SD. Pooled, Cr concentrations were significantly higher than BASE at FU1 only.

fDPD/Cr: There was no significant main effect of *Time* (P = 0.327) and no significant *Group* x *Time* interaction (P = 0.323) for fDPD/Cr concentrations (Figure 5.6, Panel B).

Cr: There was a significant main effect of *Time* (P < 0.001) but no significant *Group* x *Time* interaction (P < 0.220) for Cr concentrations. Pooled, mean concentrations were significantly (P < 0.01) higher than BASE at FU1 with concentrations increased by 85 ± 220% and 126 ± 121% in LONG and SHORT (Figure 5.6, Panel C). Mean concentrations were not significantly different from BASE at FU2 or thereafter. There was considerable variation in Cr levels both above and below BASE levels in individual subjects. At 0800 h on Day 5 in LONG (after subjects had completed one bout of exercise) concentrations ranged from 60% to 389% of BASE levels whereas the range in SHORT was 60% to 317%, despite no exercise having been completed (Figure 5.7, Panels A and B). At FU1, where two bouts of exercise had been completed in both conditions, Cr levels ranged from 94% to 806% and 71% to 470% of BASE levels in LONG and SHORT, with 4 subjects in LONG and 1 subject in SHORT showing lower Cr levels than at BASE. From FU2 to FU4 Cr levels ranged from 11% to 539% of BASE levels across both groups.

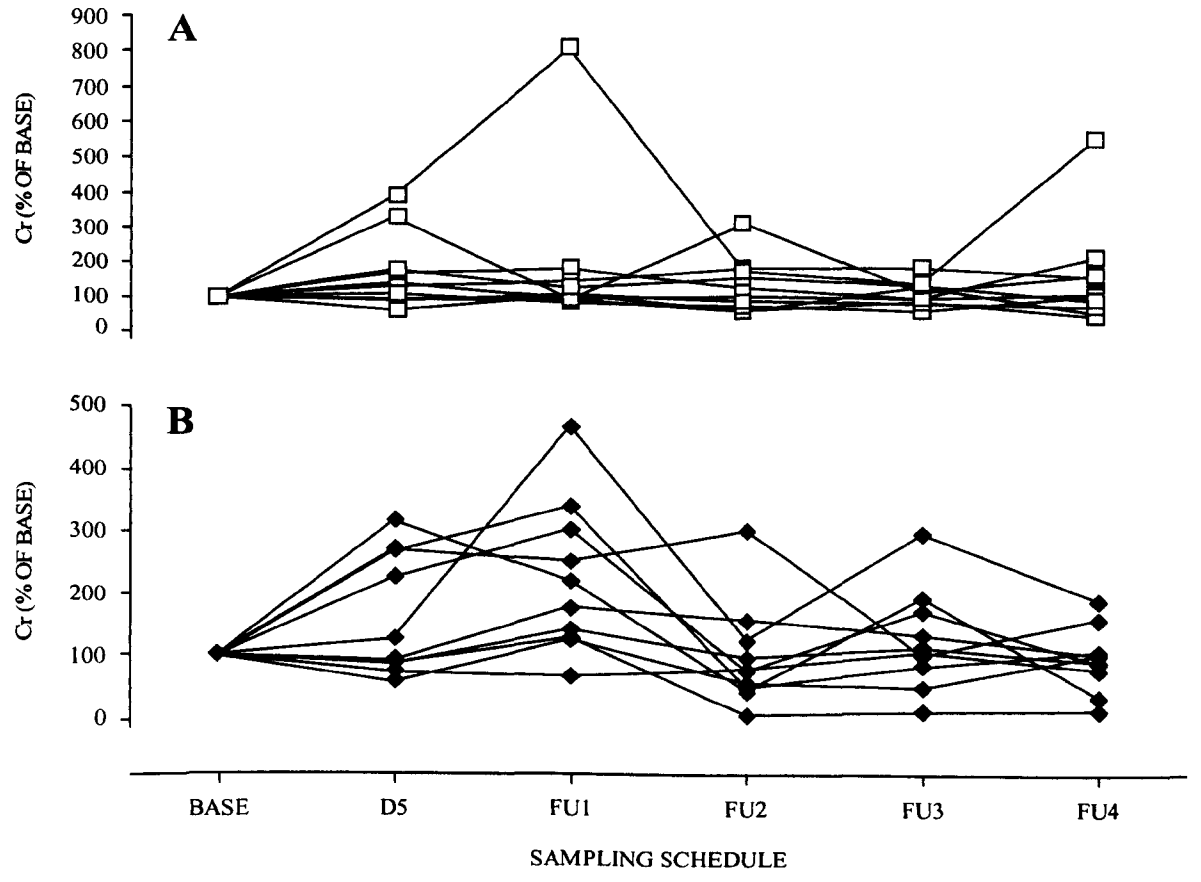

Figure 5.7. Percentage change in BASE concentrations of Cr in individual subjects in the LONG (Panel A) and SHORT (Panel B) at Day 5 (D5) and on the four follow up days (FU1 – FU4). Samples were second voids collected between 0730 h and 0800 h after an overnight fast. Values are mean ± 1SD. Different scales are used for clarity.

fPYD output: There was a significant main effect of *Time* ($P < 0.05$) but no significant *Group* x *Time* interaction ($P = 0.370$) for fPYD output. Pooled, mean output showed small, non-significant fluctuations both above and below baseline from D5 to FU3 but were reduced approximately 20% in both groups at FU4 resulting in significantly ($P < 0.05$) lower concentrations compared with BASE (Figure 5.8, Panel A).

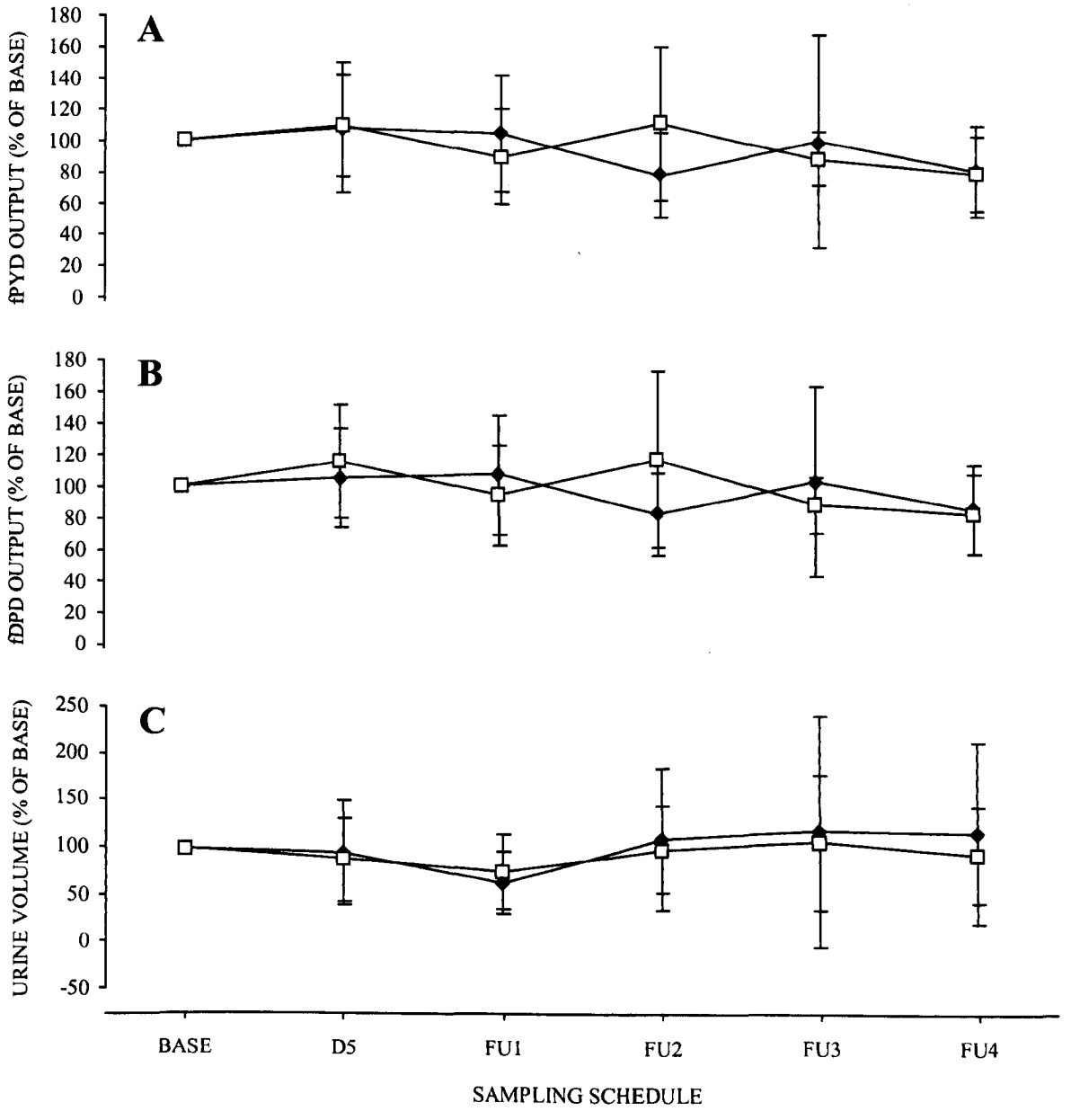

Figure 5.8. Percentage change from BASE of fPYD (Panel A) and fDPD (Panel B) output and urine volume (Panel C) at Day 5 (D5) and on the four follow up days (FU1 – FU4) in the LONG (open squares) and SHORT (filled diamonds) conditions. Values are mean ± 1SD. All samples collected between 0730 h and 0800 h after an overnight fast. fPYD or fDPD output (nmol) = fPYD or fDPD ($nmol \cdot L^{-1}$) x urine volume (L). Pooled, fPYD output and fDPD outputs were significantly lower than BASE at FU4 only. Pooled, urine volumes were significantly lower than BASE at FU1 only.

fDPD output: There was a significant main effect of *Time* ($P < 0.05$) but no significant *Group* x *Time* interaction ($P = 0.292$) for fDPD output. Changes in fDPD output paralleled those of fPYD output with pooled, mean output significantly ($P < 0.05$) lower than BASE at FU4 (Figure 5.8, Panel B).

Urine volume: There was a significant main effect of *Time* ($P < 0.05$) but no significant *Group* x *Time* interaction ($P = 0.816$) for urine volume. Pooled, mean urine volume was not significantly different from BASE on D5 but was significantly ($P < 0.001$) lower at FU1 with concentrations reduced to 73 ± 40% and 61 ± 34% of BASE levels in LONG and SHORT (Figure 5.8, Panel C). At this time point, three subjects showed increases in urine volume of 30% or more from BASE and, from FU2 to FU4, urine volumes ranged from 14 to 301% of BASE across both groups, with considerably variation both across and within individual subjects (Figure 5.9, Panels A and B).

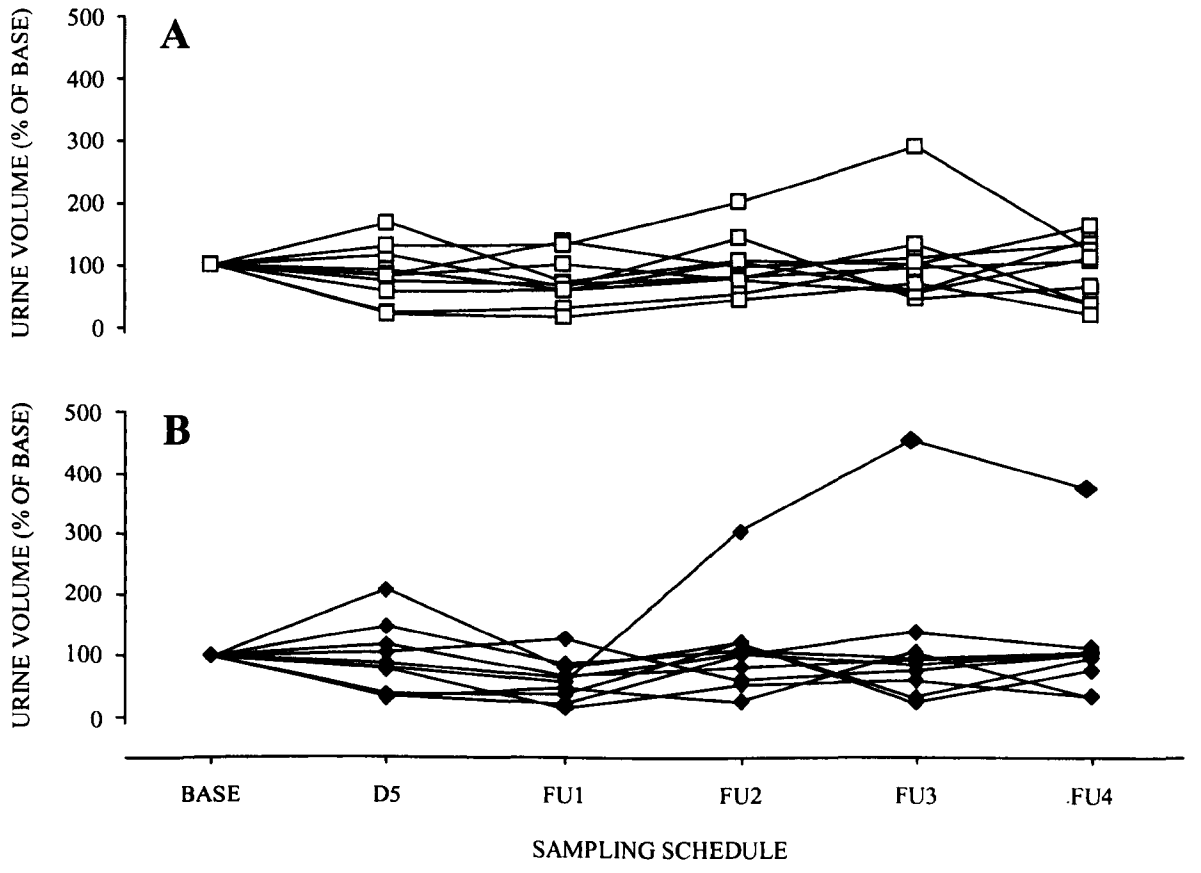

Figure 5.9. Percentage change in BASE in urine volume in individual subjects in the LONG (Panel A) and SHORT (Panel B) at Day 5 (D5) and on the four follow up days (FU1 – FU4). Samples collected between 0730 h and 0800 h after an overnight fast. Values are mean ± 1SD.

5.3.8 OPG

OPG: There was no significant differences (P = 0.872) between the changes in OPG from pre- to immediately post-exercise during the four exercise bouts (Figure 5.10).

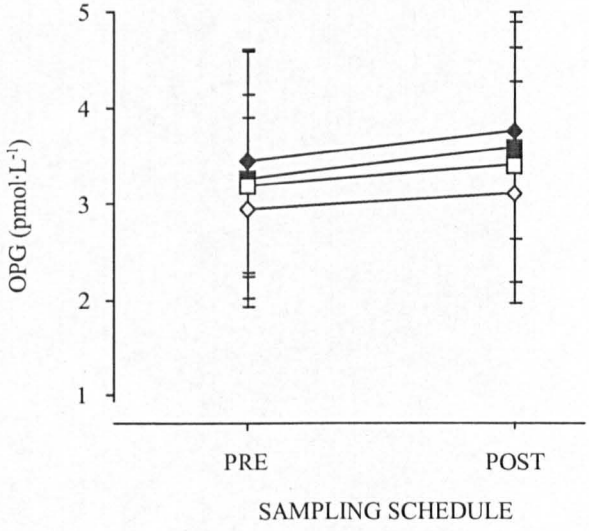

Figure 5.10. Change in OPG from pre- to immediately post-exercise in L-ExA (filled squares), L-ExB (open squares), S-ExA (open diamonds), S-ExB (filled diamonds). Values are mean ± 1SD.

When data was examined from 1415 h on Day 5 to FU4, there was a significant main effect of *Time* (P < 0.05) but no significant *Group* x *Time* interaction (P = 0.949) for OPG concentrations. Pooled, mean concentrations were increased 10% from BASE immediately after exercise and decreased 20% at FU3 and FU4 but no individual time points were significantly different from BASE (Figure 5.11).

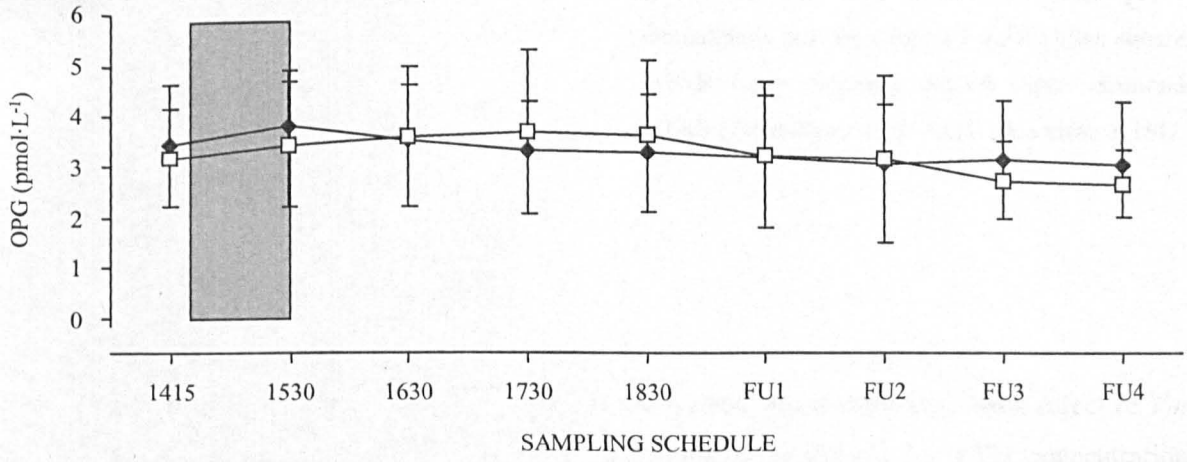

Figure 5.11. OPG concentrations before (1415 h) and after (1530 h – 1830 h) exercise bout B (ExB) on Day 5 and on four follow-up days (FU1 – FU4) in the LONG (open squares) and short (filled diamonds) conditions. Grey box denotes ExB. Values are mean ± 1SD.

5.3.9 Calcium Metabolism

PTH: There was no significant differences (P = 0.553) between the changes in PTH from pre- to immediately post-exercise during the four exercise bouts (Figure 5.12, Panel A).

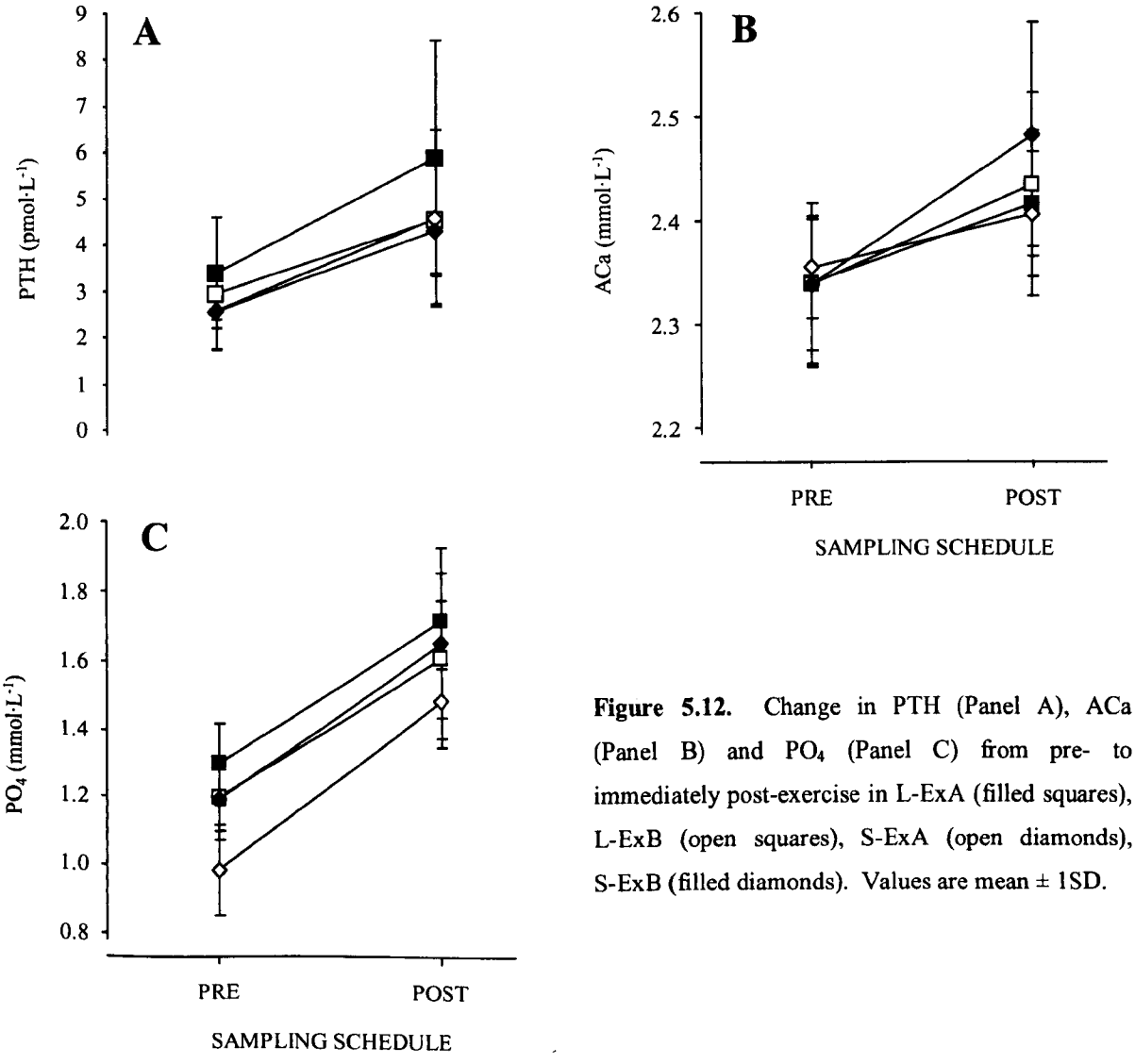

Figure 5.12. Change in PTH (Panel A), ACa (Panel B) and PO_4 (Panel C) from pre- to immediately post-exercise in L-ExA (filled squares), L-ExB (open squares), S-ExA (open diamonds), S-ExB (filled diamonds). Values are mean ± 1SD.

When data was examined from 1415 h on Day 5 to FU4, there was a significant main effect of *Time* (P < 0.01) but no significant *Group* x *Time* interaction (P = 0.543) for PTH concentrations. Pooled, mean concentrations were significantly (P < 0.001) increased immediately after exercise where concentrations were 55 ± 30% and 69 ± 40% higher than at 1415 h in LONG and SHORT (Figure 5.13, Panel A). Concentrations declined rapidly thereafter and were significantly (P < 0.05) reduced by 10 to 15% at 1630 h compared with 1415 h. This reduction was transient, with concentrations not significantly different from 1415 h at 1730 h or 1830 h. Although mean concentrations were significantly (P < 0.01) increased from 1415 h at FU2 and FU3, concentrations at

this time point (3.3 and 3.6 pmol·L^{-1}) were similar to those at BASE (3.5 and 3.5 pmol·L^{-1}) in LONG and SHORT.

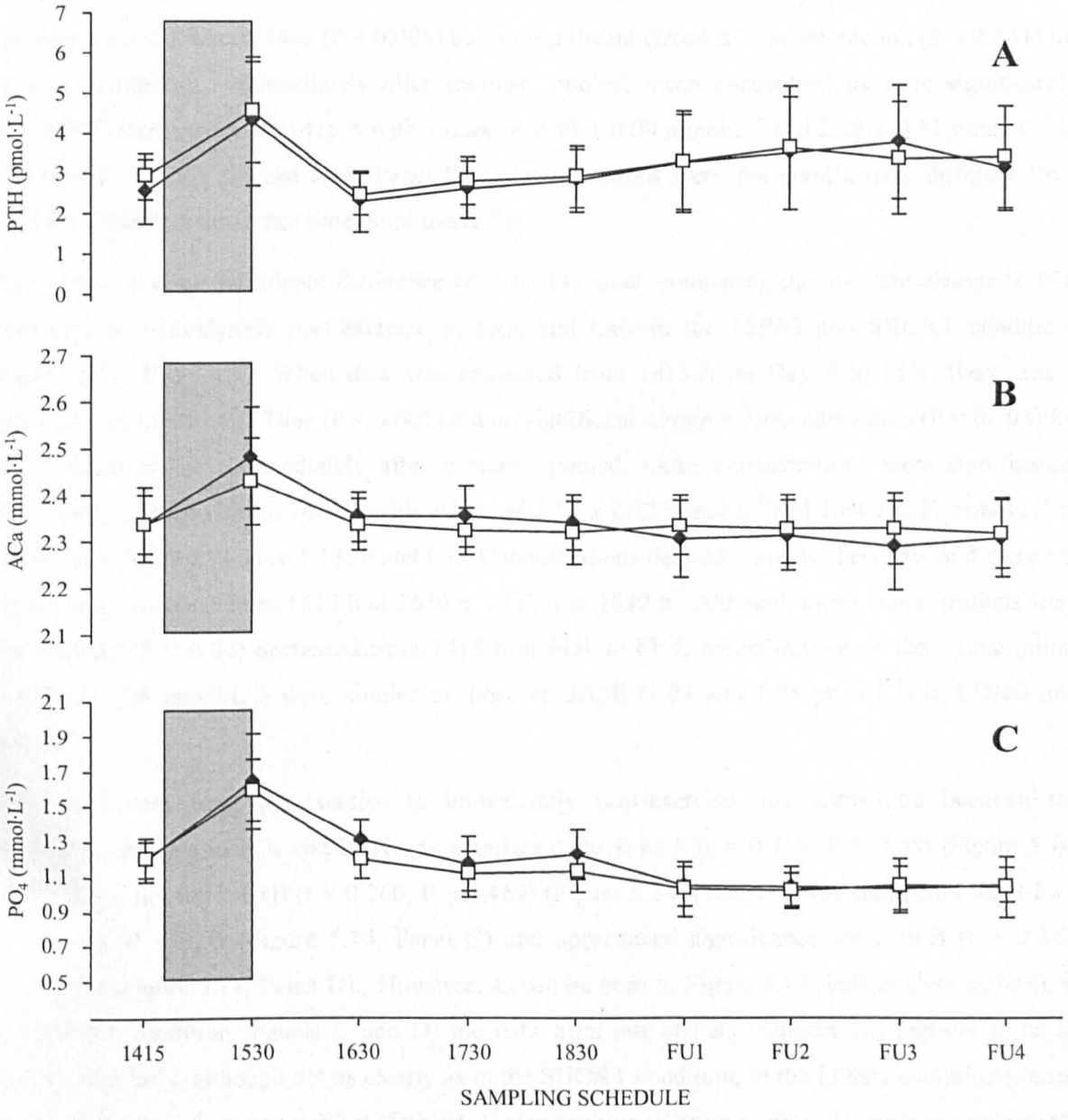

Figure 5.13. PTH (Panel A), ACa (Panel B) and PO_4 (Panel C) concentrations before (1415) and after (1530 h – 1830 h) exercise bout B (ExB) on Day 5 and on four follow-up days (FU1 – FU4) in the LONG (open squares) and SHORT (filled diamonds) conditions. Grey box denotes ExB. Values are mean ± 1SD. Pooled, PTH concentrations were significantly higher than 1415 h at 1530 h, significantly lower at 1630 h and significantly higher at FU2 and FU3. Pooled, ACa concentrations were significantly higher than 1415 h at 1530 h only. Pooled, PO_4 concentrations were significantly higher than 1415 h at 1530 h and significantly lower at FU-FU4.

ACa: There was no significant difference ($P = 0.055$) when comparing the absolute change in ACa from pre- to immediately post-exercise in ExA and ExB in the LONG and SHORT conditions (Figure 5.12, Panel B). When data was examined from 1415 h on Day 5 to FU4, there was a significant main effect of *Time* ($P < 0.001$) but no significant *Group* x *Time* interaction ($P = 0.334$) for ACa concentrations. Immediately after exercise, pooled, mean concentrations were significantly ($P < 0.001$) increased from 1415 h with values of 2.43 ± 0.09 mmol·L^{-1} and 2.48 ± 0.11 mmol·L^{-1} in LONG and SHORT (Figure 5.13, Panel B). Concentrations were not significantly different from 1415 h at 1630 h or any other time point thereafter.

PO_4: There was no significant difference ($P = 0.711$) when comparing the absolute change in PO_4 from pre- to immediately post-exercise in ExA and ExB in the LONG and SHORT conditions (Figure 5.12, Panel C). When data was examined from 1415 h on Day 5 to FU4, there was a significant main effect of *Time* ($P < 0.001$) but no significant *Group* x *Time* interaction ($P = 0.703$) for PO_4 concentrations. Immediately after exercise, pooled, mean concentrations were significantly ($P < 0.001$) increased from 1415 h with values of 1.60 ± 0.17 mmol·L^{-1} and 1.64 ± 0.27 mmol·L^{-1} in LONG and SHORT (Figure 5.13, Panel C). Concentrations declined rapidly thereafter and were not significantly different from 1415 h at 1630 h, 1730 h or 1830 h. Although mean concentrations were significantly ($P < 0.01$) decreased from 1415 h at FU1 to FU4, concentrations at these time points (1.02 and 1.04 mmol·L^{-1}) were similar to those at BASE (1.04 and 1.08 pmol·L^{-1}) in LONG and SHORT.

When measured from pre-exercise to immediately post-exercise, the correlation between the percentage changes in PO_4 and PTH was significant for L-ExA ($r = 0.717$, $P < 0.05$) (Figure 5.14, Panel A) but not for L-ExB ($r = 0.260$, $P = 0.469$) (Figure 5.14, Panel B), was significant for S-ExA ($r = 0.711$, $P < 0.05$ (Figure 5.14, Panel C) and approached significance for L-ExB ($r = 0.622$, $P = 0.055$) (Figure 5.14, Panel D). However, as can be seen in Figure 5.14 (indicated by arrows), in the SHORT condition (Panels C and D) the data from one subject (Subject 10) appears to be an outlier. Similarly, although not as clearly as in the SHORT condition, in the LONG condition (Panels A and B) the data from one subject (Subject 7) also appears to be an outlier. In order to evaluate the effects of these possible outliers on correlation values, the data from these subjects were removed and the correlations repeated. With outliers removed, correlations between changes in PO_4 and PTH were not significant for either exercise bout in the LONG and SHORT condition (L-ExA: $r = 0.430$, $P = 0.248$; L-ExB: $r = 0.324$, $P = 0.395$; S-ExA: $r = 0.108$, $P = 0.787$; S-ExB: $r = -0.307$, $P = 0.422$). These revised correlations are shown in Appendix B.

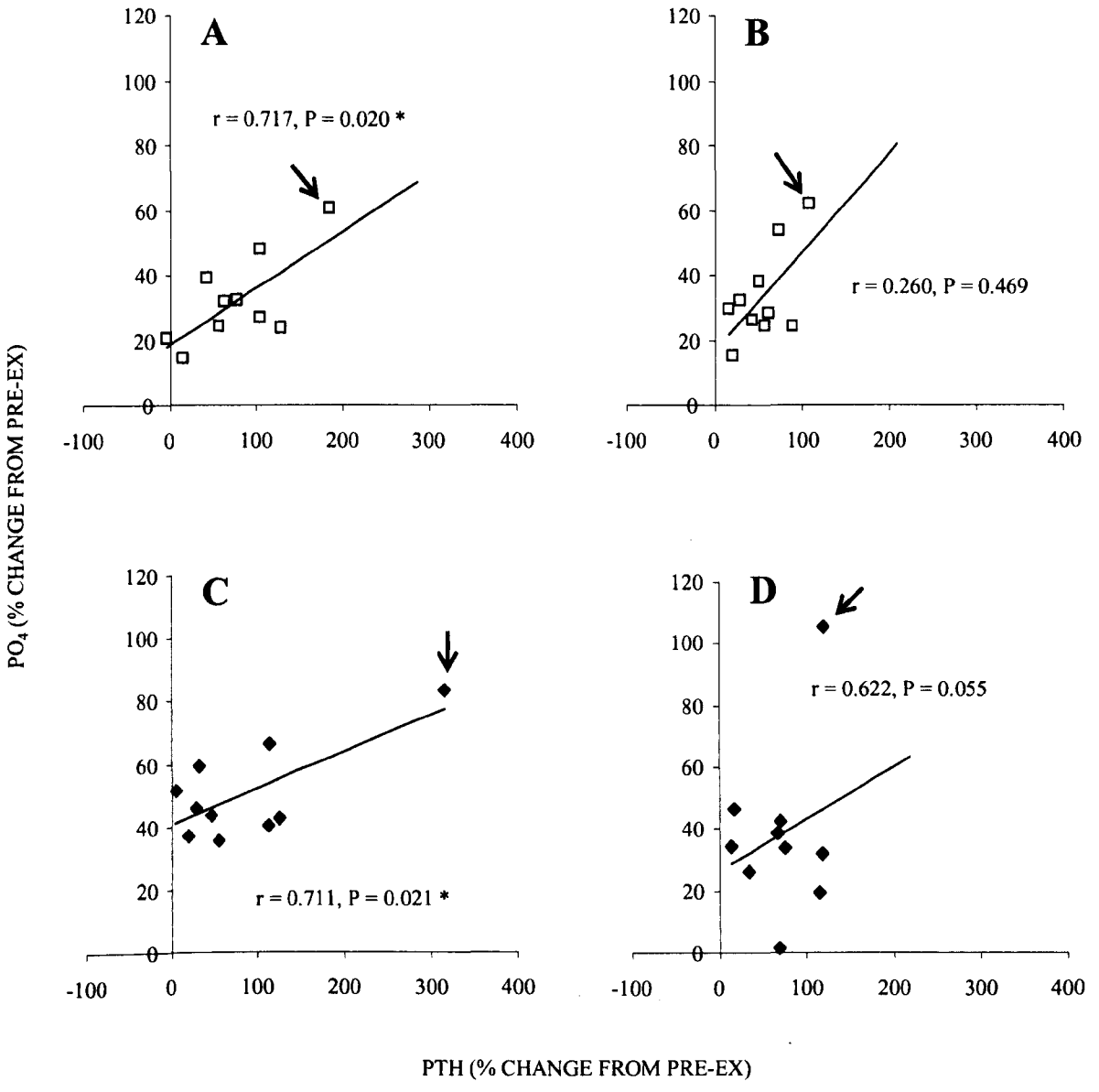

Figure 5.14. Relationship between the percentage changes in PO_4 and PTH concentrations measured from pre-exercise to immediately post-exercise during L-ExA (Panel A), L-ExB (Panel B), S-ExA (Panel C) and S-ExB (Panel D). * significant ($P < 0.05$). Arrows indicate possible outliers (Subject 7 in the LONG condition and Subject 10 in the SHORT condition).

5.4 Discussion

The main findings from this study are that 1) when measured from 1-4 days post-exercise, two, 60 min bouts of running at 65% VO_{2max} separated by either 23 h or 3 h have no significant effect on concentrations of blood-based markers of bone resorption and formation, or fPYD/Cr and fDPD/Cr; 2) over the same sampling time period, fPYD and fDPD output show a small but significant reduction on the fourth day post-exercise but there was no effect of recovery duration and; 3) reducing the recovery duration from 23 h to 3 h between two bouts of exercise has no effect on the responses of OPG, PTH, ACa or PO_4 to the second bout of exercise.

The lack of any change in β-CTX and P1NP with either protocol was unexpected. Several studies report increased P1CP and 1CTP three days after light jogging (Thorsen *et al.*, 1997) and increased P1CP but not 1CTP three days after a 3 h run at 12 $km \cdot h^{-1}$ (Langberg *et al.*, 1999) and a competitive marathon (Langberg *et al.*, 2000), but there is a lack of convincing evidence that exercise results in increases in specific markers of bone turnover on the days following exercise.

P1NP is unaffected 24 h after 30 min of brisk walking on a treadmill with and without a 5 kg load (Tosun *et al.*, 2006). P1NP and β-CTX are decreased with low intensity exercise and increased with high intensity exercise 24 h after a 60 min bout of cycling (Herrmann *et al.*, 2007) although this study did not control either the time of day of blood sampling or the nutritional status of subjects, both of which are known to have significant influence on the concentrations of bone turnover markers, particularly β-CTX (Wichers *et al.*, 1999; Clowes *et al.*, 2002a; Henriksen *et al.*, 2003). Likewise, Kerschan-Schindl *et al.* (2009) report a 40% increase in β-CTX the morning after a 246 km run but failed to standardise the nutritional status of their subjects. Bone ALP is unchanged in men from 1 to 5 days after a marathon (Malm *et al.*, 1993), which is in agreement with the results in the present study.

In the present study, the only measure of the effect of a single bout of running on BTM is from the blood sample taken at 0800 h on Day 5 in the LONG condition as, prior to all other blood samples, either no exercise (0800 h on Day 5 in SHORT) or two bouts of running (all other samples) had been performed. From this blood sample there is no evidence of an effect of one, 60 min bout of running at 65% VO_{2max} on blood-based BTM when standardising the time-of-day and subjects' acute nutritional status.

It is of course possible that one, 60 min bout of running at 65% VO_{2max} has no effect at all on bone turnover markers. With cycling, 60 min at 80% VO_{2max} increases β-CTX concentrations (Guillemant *et al.*, 2004) and 60 min at 95% and 110%, but not 75%, of AT increases β-CTX and OC, with P1NP increased only at 110% of AT (Herrmann *et al.*, 2007). Additionally, β-CTX and OC are increased with 50 min of cycling at 115% but not 85% of VT (Maïmoun *et al.*, 2006). There is no comparative data for acute running but based on the data described above, it is possible that the

intensity of the exercise used in the present study was not sufficient to stimulate bone turnover markers.

The lack of any change in β-CTX from 1-4 days post-exercise is in contrast to Study I. Given the exercise protocols, particularly that in SHORT where the total duration of exercise (2 h) was similar to the average exercise duration in Study I, some effect on β-CTX might have been anticipated. Despite the similar total exercise duration, however, there are clearly a number of differences between the study designs and exercise protocols, which might have influenced the different outcomes. Physical characteristics of the subjects in the two studies are unlikely to play a role, while the β-CTX response was unaffected by training status in Study I and the range of physical fitness of subjects in Study II was similar to that in the RA and *et* groups in Study I.

The complex and exhaustive nature of the exercise protocol in Study I makes the identification of individual components that might be responsible difficult. In Study I, however, IEE was performed at 70% VO_{2max}, whereas all exercise in Study II was performed at 65% VO_{2max}. As several studies suggest there is an exercise intensity threshold below which β-CTX concentrations do not increase (Maïmoun *et al.*, 2006; Herrmann *et al.*, 2007), this small difference in exercise intensity might explain the lack of change in β-CTX in Study II. However, the effects of exercise intensity on the β-CTX response to acute running have not been investigated.

A further possibility is that differences in pre-exercise nutritional status was a factor. The ingestion of food results in a rapid suppression of β-CTX concentrations that lasts for several hours (Henriksen *et al.*, 2003). It is possible that this suppression of resting concentrations might also attenuate the increase in β-CTX during subsequent exercise. This idea has never been investigated but the principle is supported by studies that show that calcium ingestion has a similar effect to that of food on resting β-CTX concentrations (Guillemant *et al.*, 2000; Zikán *et al.*, 2001) and the abolition of the increase in β-CTX during subsequent cycling (Guillemant *et al.*, 2004).

The present study appears to be the first study to examine the effect of recovery duration between two bouts of exercise on bone turnover markers. There are, however, several studies that have examined bone marker responses to bouts of exercise on several consecutive days. Zanker and Swaine, (2000) report no change in P1NP, OC or urinary NTX or fDPD following one, 60 min bout of running (65%-85% VO_{2max}) on three consecutive days, but only if energy intake was adjusted to account for the increase in energy expenditure associated with exercise. When energy intake was restricted by 50% however, P1NP concentrations were reduced by 20%.

From blood and urine samples taken before and after a 5 day experimental period, during which subjects expended 15 kcal·kg LBM^{-1}·day^{-1} by walking at 70% VO_{2max} and had their energy intake reduced in a dose-dependent manner, Ihle and Loucks, (2004) report a progressive decrease in P1CP and OC with decreasing energy availability. At the lowest level of energy availability, urinary NTX

also increased, although, like Zanker and Swaine, (2000), when subjects were kept in energy balance, there was no change in BTM.

In the present study, in order to minimise any changes in bone turnover resulting from alterations in energy availability (Zanker and Swaine, 2000; Ihle and Loucks, 2004), a standardised diet was provided on Days 4 and 5 to account for the increased energy expenditure due to exercise. As running at 9 $km \cdot h^{-1}$ has an energy cost of approximately 11 $kcal \cdot min^{-1}$, this supplement was estimated to be 660 kcal (Åstrand and Rodahl, 1986). In relation to the actual running speeds used in the study, 9 $km \cdot h^{-1}$ was towards the lower end of the range (8.7 to 12.3 $km \cdot h^{-1}$). Based on mean inspired O_2 and expired CO_2 values from the four exercise bouts, and the calculations of Frayn, (1983) the mean energy expended during exercise was actually 862 ± 95 $kcal \cdot h^{-1}$ (range, 729 to 1019 $kcal \cdot h^{-1}$). In addition, the supplement was averaged over two days in both conditions rather than all being provided on Day 5 in SHORT when both bouts of exercise were completed. Although the actual energy cost of the exercise protocols was somewhat greater than the quantity of energy provided in the supplement, there was no significant change in body mass from Day 4 to Day 9 in either condition. Although a change in body mass is likely not a sensitive measure of energy balance over such a short period of time, together with the lack of a significant decrease in P1NP and bone ALP concentrations, this suggests that the energy provided in the diet was sufficient to prevent a marked energy deficit. Although there are a number of differences between the design of the present study and those of Zanker and Swaine, (2000) and Ihle and Loucks, (2004), the present results appear consistent with these previous investigations by showing that in subjects who have an appropriate energy intake, serum markers of bone turnover are unaltered in response to repeated bouts of exercise.

As with the blood-based BTM, there was also no change in Cr-corrected fPYD and fDPD in the present study. The Cr-corrected results are in contrast to those reported by Welsh *et al.* (1997) who report a 50% increase in both fPYD and fDPD the day after a 30 min walk at 60% of HR_{max}. The significant increase in urinary Cr evident at FU1 in the present study might have masked any changes in fPYD and fDPD. Welsh *et al.* (1997) did not measure any serum markers of bone resorption to corroborate their findings for fPYD and fDPD, and the lack of any effect on β-CTX in the present study, supports the unchanged fPYD and fDPD levels in response to the two exercise schedules.

In response to the marked and sustained increase in Cr concentrations in Study I, pyridinoline output was also calculated in the present study by multiplying pyridinoline concentration by SMV urine volume. In contrast to the Cr-corrected results, there was a significant decrease of 20% in both fPYD and fDPD at FU4. This finding appears, in part, consistent with the findings Ashizawa *et al.* (1998) who report that fDPD output in 24 h samples are reduced by 22% and 27% at 1 and 3 days after a single bout of high intensity, resistance exercise.

Using urine volume rather than Cr to 'correct' urinary markers avoids problems related to both the day-to-day variability in Cr excretion (Cryer and Sode, 1970; Greenblatt *et al.*, 1976) and the increase in Cr associated with exercise (Refsum and Strömme, 1974; Srivastava *et al.*, 1957). Whilst the variability introduced by Cr normalisation might be acceptable for large population studies, for smaller subject numbers or the evaluation of individuals, this level of variability could significantly affect the interpretation of results.

Despite the theoretical benefit of calculating pyridinoline output, there have been few studies comparing urinary pyridinolines expressed as 'per-creatinine' to their expression 'per-day'. Smith *et al.* (2004) showed that correlations between the urinary content of NTX, fPYD and fDPD in two, 24 h samples tended to be similar whether expressed as per-creatinine or per-day (0.51-0.76 *vs* 0.52-0.74). However, they did observe a lower correlation for urinary Cr when expressed as per-litre compared with per-day (0.34 *vs* 0.50).

Smith *et al.* (2004) do not report the correlation between the volume of urine produced in the two, 24 h samples they collected, so it is not possible to compare their day-to-day variability with that of creatinine, although there is considerable evidence of large variability in the urine volume in 24 h samples collected from the same individual on different days (Jackson, 1966; Elkins *et al.*, 1974; Duke, 1998; Jones *et al.*, 2007; Marco *et al.*, 2008). Marco *et al.* (2008) report large variations in urine volume in successive spot samples collected during the same 24 h period, although this might be expected due to fluctuations in food and fluid intake throughout the course of the day. By standardising the spot sample (*e.g.* a SMV) it is possible that this variability would be reduced although there do not appear to have been any investigations that have examined the day-to-day variability in the volume of SMV urine samples. As the present study did not include a non-exercising control group it is not possible to examine the correlation between urine volume in two, consecutive SMV urine samples in the rested state. However, it is clear from the individual data in the present study that there is considerable variation in urine volume in consecutive SMV urine samples in an exercising population.

In the present study, mean urine volumes in SMV samples were significantly reduced at FU1. In previous studies, mean urine volumes may be reduced in the hours immediately after exercise (Ashizawa *et al.*, 1997). In the longer-term, findings are inconsistent with urine flow rates elevated from 2 to 5 days after a marathon in 24 h urine samples (Irving *et al.*, 1986) or unchanged up to 3 days after resistance exercise (Ashizawa *et al.*, 1998) or 5 days after a 56 km run (Irving *et al.*, 1990) although differences in voluntary fluid intake in these studies is likely to be a factor. In the study of Irving *et al.* (1990), despite no significant changes, mean urine production (and the magnitude of the standard deviation) fluctuated considerably over the 5 post-exercise days with the lowest mean levels and smallest standard deviation on the first day after the race. Similarly, despite reporting no significant change in urine volume, Ashizawa *et al.* (1998) report urine volumes of 1317 ± 106 mL on

the exercise day, and 1044 ± 508 mL and 1141 ± 123 mL on the first and third day post-exercise, suggesting both a tendency for lower urine volumes following exercise and considerable individual variation.

When calculating pyridinoline output, a decrease in urine volume could mask a genuine increase in pyridinoline levels. It is therefore possible that the decrease in urine volume at FU1 might have masked a true increase in pyridinolines although there was no change in β-CTX at this time point output. Similarly, in the study of Ashizawa *et al.* (1998), the reduced fDPD output at 1 and 3 days post-exercise might be related to the tendency for lower urine volumes. However, when mean fPYD and fDPD output was decreased at FU4 in the present study there was no change in urine volume. It is therefore possible that the decrease in pyridinoline output is indeed a genuine effect of the two exercise protocols although again, β-CTX was not significantly different from baseline at this time point.

This is the first study to measure the OPG response to repeated bouts of exercise and also the first to examine the effect of different recovery durations from prior exercise. There was a small but significant increase in plasma OPG concentrations when measured immediately after exercise but reducing the recovery duration between two bouts of running from 23 h to 3 h had no effect on changes in OPG. There was a marked increase in OPG during FD in Study I, which was identical to the exercise bouts used in the present study yet increases in OPG were much less. As three of the four exercise bouts were performed in the afternoon, the increase in OPG might have been attenuated by declining concentrations due to its circadian rhythm (Joseph *et al.*, 2007) although Ziegler *et al.* (2005) report no increase in OPG after a running race that was of greater length (15.8 km) and duration (1.26 ± 0.19 h) than exercise bouts in the present study (8.7-12.3 km and 60 min). Currently, the reason for these different responses is unclear. As peak OPG concentrations in Study I occurred at the end of exhaustive exercise and OPG is increased 100% and 150% after a marathon and a 246 km run (Ziegler *et al.*, 2005; Kerschan-Schindl *et al.*, 2009), it is possible that the OPG response to exercise may be less marked and more variable with a more modest exercise stimuli.

Increased OPG concentrations in conditions associated with increased bone resorption (Yano *et al.*, 1999; Grigorie *et al.*, 2003; Misra *et al.*, 2003) have been interpreted as a compensatory response. The only study of exercise to measure both OPG and β-CTX (Kerschan-Schindl *et al.*, 2009) reports increases in both markers the morning after a 246 km running race which might also indicate a compensatory effect. In the present study BTM were only measured in early morning fasted samples and neither β-CTX nor OPG concentrations were increased on the four follow-up days making it difficult to draw any further conclusions about this relationship.

Mean PTH concentrations were increased with all exercise bouts by between 55 and 85%, returning to baseline by 1 h post exercise. This compares well with previous studies that have examined changes in PTH with an acute bout of running of up to 60 min duration and report increases in PTH of between 20 and 80% at intensities ranging from approximately 50 to 85% (Nishiyama *et al.*, 1988; Salvesen *et al.*, 1994; Bouassida *et al.*, 2003).

With PTH basal concentrations declining in the late morning (Fuleihan *et al.*, 1997; Ahmad *et al.*, 2003) the increase in PTH in ExA in SHORT is likely a true effect of exercise although it is not possible to rule out a partial contribution of the circadian rhythm to the increase in PTH during the remaining exercise bouts performed in the afternoon. That said, the magnitude of the increase in PTH during these bouts is greater than the change that would be expected due to the circadian rhythm alone over the same period (Fuleihan *et al.*, 1997; Ahmad *et al.*, 2003).

Previously, only one study has examined the PTH response to two bouts of endurance exercise performed on the same day (Bouassida *et al.*, 2003). In this study, subjects performed 21 min of running at 70% VO_{2max} and 21 min of running at 85% VO_{2max} either consecutively of separated by a 40 min rest. With continuous exercise, PTH concentrations increased 10-15% after the first half of exercise and 85% at the end of exercise. In contrast, with intermittent exercise, PTH was increased after the first session but there was no further increase in PTH at the end of the second bout, suggesting that a prior bout of running attenuates the PTH response to a second bout. The magnitude of this attenuation is particularly surprising because of the marked effect of continuous exercise on PTH and because higher exercise intensities are associated with marked increases in PTH (Guillemant *et al.*, 2004; Maïmoun *et al.*, 2006). In contrast, in the present study, when two bouts of exercise of greater duration but lower intensity are separated by a 3 h recovery period, the PTH response, both in magnitude and duration, is preserved.

As in Study 1, PTH rapidly returned to baseline with the termination of exercise and although mean PTH concentrations were significantly increased from pre-ExB levels at FU2 and FU3, they were similar to those at BASE suggesting no long-term effect of either protocol on PTH. This transient (less than 2 h) increase in PTH is consistent with the change seen with daily injections of PTH that result in bone formation (Neer *et al.*, 2001). Several previous studies report transient increases in PTH with acute exercise (Rudberg *et al.*, 2000; Guillemant *et al.*, 2004; Tosun *et al.*, 2006) but none provide convincing evidence of an anabolic effect with bone markers either unchanged (Guillemant *et al.*, 2004; Tosun *et al.*, 2006) or transiently increased during exercise only (Rudberg *et al.*, 2000). However, none of these studies measured bone formation markers beyond 24 h post-exercise.

In the present study, despite two transient increases in PTH with exercise in both experimental conditions, there was no effect on bone formation markers measured up to four days after the second bout of exercise. However, if, as suggested by the results of Study I, PTH was elevated continuously

throughout FD and IEE, this might explain the different changes in β-CTX between Study I and the present study, as a continuous increase in PTH concentrations also increases β-CTX (Lee *et al.*, 2006). It is possible that, despite an approximately similar magnitude and total duration of increased PTH, the intermittent nature of the increases in PTH in the present study was not sufficient to stimulate either bone formation or bone resorption.

A transient decrease in PTH from pre-exercise concentrations was seen in the early post-exercise period although the magnitude and duration was less marked than that in Study I. As the PTH circadian rhythm increases basal concentrations in the afternoon, the significant reduction in PTH concentrations is likely a genuine effect of exercise. Interestingly, unlike in Study I, PTH concentrations were reduced despite no evidence of increased post-exercise calcium concentrations.

As in Study I, in the present study serum calcium was increased with exercise suggesting that the increase in PTH was not stimulated by a decrease in calcium concentrations. No measures were taken during exercise so a drop in calcium during exercise cannot be ruled out but the increase in ACa during FD in Study I and findings from previous studies (Salvesen *et al.*, 1994; Tsai *et al.*, 1997; Guillemant *et al.*, 2004) suggest this to be unlikely. The increase in ACa observed at the end of exercise might result from the mobilisation of calcium from bone by osteoclastic activity stimulated by increased PTH concentrations during exercise.

In the study of Bouassida *et al.* (2003) the insertion of a rest break, which blunted the PTH response to a second bout of exercise, also prevented a further decline in iCa that was evident during the second half of their continuous protocol. This might suggest that, at least during S-ExB, changes in iCa are unlikely to explain the preserved increase in PTH. In addition, other studies report that the increase in PTH with acute exercise occurs despite either no change (Ljunghall *et al.*, 1985; Ljunghall *et al.*, 1986; Rong *et al.*, 1997; Rudberg *et al.*, 2000) or an increase (Ljunghall *et al.*, 1984b; Henderson *et al.*, 1989) in iCa concentrations.

Metabolic acidosis is unlike to have contributed to the increase in PTH in the present study as it might do during severe exercise (Hermansen and Osnes, 1972; Sahlin *et al.*, 1976; Costill *et al.*, 1983; King *et al.*, 1985; Nevill *et al.*, 1989; Allsop *et al.*, 1990). Although blood pH wasn't measured, low (<2 $mmol \cdot L^{-1}$) post-exercise lactate concentrations make a drop in pH unlikey, while other studies report increased PTH with exercise despite no change in blood pH (Rudberg *et al.*, 2000; Guillemant *et al.*, 2004).

Catecholamine concentrations were also not measured in this study, although the significant accumulation of epinephrine and norepinephrine in the blood only occurs at exercise intensities similar to that of the lactate and ventilatory threshold (Mazzeo and Marshall, 1989; Weltman *et al.*, 1994). Again, given the low lactate levels in the present study, a significant accumulation of catecholamines in the circulation appears unlikely to mediate the increase in PTH.

Similarly to Study I, both PO_4 and PTH concentrations were increased during exercise, with PO_4 increased between 30-50% by the end of exercise. As small (< 50%) increases in the circulating PO_4 concentration are associated with increases in PTH concentrations (Silverberg *et al.*, 1986; Kärkkäinen and Lamberg-Allardt, 1996; Martin *et al.*, 2005) with changes occurring in a matter of minutes (Martin *et al.*, 2005), the increase in PO_4 might have stimulated the increase in PTH. Although significant or borderline significant correlations between changes in PO_4 and PTH were present during three of the four exercise bouts, when outliers were removed and correlations re-computed, all correlations were weaker and not significant. This suggests that other changes in PO_4 are unlikely to be mediating changes in PTH.

In conclusion, two 60 min bouts of running at 65% VO_{2max} separated by either 23 h or 3 h had no effect on serum markers of bone resorption or bone formation or on urinary pyridinolines corrected for creatinine concentrations. This suggests that, in physically-active men, who have consumed a daily diet appropriate in terms of energy content, neither schedule of repeated exercise has any adverse impact on bone turnover. In contrast, when the pyridinoline output was calculated, despite the lack of change in serum bone resorption markers, both conditions were associated with a decrease at 4 days post exercise. Although urine volume was decreased following both conditions, the decrease in pyridinolines at FU4 does not appear to result from a change in urine volume. OPG concentrations are increased with exercise but the increase to a second bout of exercise is not affected by reducing the recovery duration from a prior bout of exercise from 23 h to 3 h. Likewise, PTH, ACa and PO_4 concentrations are all transiently increased by exercise but the increase to a second bout of exercise is not affected by reduced recovery duration from a prior bout. The increase in PTH is not explained by changes in ACa or PO_4.

CHAPTER VI

STUDY III – THE EFFECT OF EXERCISE INTENSITY ON THE METABOLIC RESPONSE OF BONE TO ACUTE RUNNING

6.1 Introduction

In Study I, a bout of exhaustive, running exercise was associated with an increase in β-CTX but not P1NP or bone ALP for four days following exercise. This indicates an alteration to the bone remodelling balance, favouring increased bone resorption in the days following exercise. However in Study II there was no change in markers of bone resorption or formation measured over the same time frame following two, 1 h bouts of running separated by either 23 h or 3 h, suggesting no negative alterations to the bone remodelling balance. The lack of any effect on bone turnover was surprisingly, particularly in the SHORT condition where subjects performed two, 60 min bouts of running at 65% VO_{2max} on the same day. Although there were a number of differences between the exercise protocols, in Studies I and II the total exercise duration was not dissimilar with the mean total exercise duration in Study 1 being approximately 2 h (116 ± 26.8 in RA and 133.7 ± 14.3 min in ET). One factor that was different however, was cardiovascular exercise intensity. In Study I, the first hour of exercise was performed at 65% VO_{2max} but the remainder of exercise was performed at 70% VO_{2max}. This was in contrast to Study II, where all exercise was conducted at 65% VO_{2max}.

Animal studies show that mechanical strains induced by loading must reach a specified magnitude before an osteogenic effect is initiated. Once this threshold is exceeded, the bone formation response is correlated positively with peak strain magnitude (Chow *et al.*, 1993; Rubin *et al.*, 1985; Turner *et al.*, 1994). Consistent with these findings, high-impact activities have been suggested to be effective in improving femoral neck BMD in pre-menopausal women (Wolff *et al.*, 1999) whilst intervention studies of brief but regular impact exercise such as jumping and hoping can significantly can also have positive effects at the hip (Bailey and Brooke-Wavell, 2010; Bassey and Ramsdale, 1994; Kato *et al.*, 2006).

Since it is impractical to directly assess bone strain in free-living humans, it has been suggested that peak acceleration might be a reasonable estimate of peak bone strain and thus act as a surrogate measure (Vainionpää *et al.*, 2006). Indeed, studies in physically-active humans that indicate that BMD is associated with peak accelerations during exercise (Gremion *et al.*, 2004; Dériaz *et al.*, 2010), while physical activity interventions must generate accelerations that exceed a certain threshold in order to be osteogenic (Jämsä *et al.*, 2006; Vainionpää *et al.*, 2006; Vainionpää *et al.*, 2007). Animal studies also indicate that bone formation is proportional to strain rate (Mosley *et al.*, 1998; Turner *et al.*, 1995). Like bone strain, it is also impractical to measure strain rate in humans during physical activities, but using the slope of the acceleration signal as a measure of loading rate (and thus

an estimate of strain rate), Heikkinen *et al.*, (2007) report that, like acceleration levels, acceleration slopes must also exceed a given threshold to be osteogenic.

Despite the apparent benefits of short duration, high impact exercise on bone, many people partake in longer duration, endurance exercise such as running, cycling and swimming, be it for recreational, competitive or occupational (*e.g.* the military) purposes. Such activities are also be characterised in terms of their 'intensity' using relative measures of cardiovascular strain such as percentage of maximum oxygen uptake (VO_{2max}), lactate threshold (LT) or heart-rate reserve (HRR). Importantly, however, although all these modes of exercise will, to a varying degree, impose mechanical loads on bone either by muscle contraction or gravity, such measures of cardiovascular exercise intensity cannot be used interchangeable with those used in relation to mechanical loads on bone.

Although weight-bearing in nature, endurance running has been associated with deleterious effects on bone in some populations including reduced spinal BMD in endurance runners (Bilanin *et al.*, 1989; Hind *et al.*, 2006) and stress fractures (SFx) in both runners (Bennell *et al.*, 1996) and military recruits (Lappe *et al.*, 2008). Both endurance runners and recruits are regularly exposed to high cardiovascular intensity exercise while military recruits also frequently perform 'common' physical training sessions during which individual relative exercise intensities range from 53% to 73% of HRR (Rayson *et al.*, 2001). It is recruits with the lowest aerobic fitness – who will experience the higher relative cardiovascular exercise intensities during these activities – that have an increased risk of SFx (Shaffer *et al.*, 1999; Välimäki *et al.*, 2005). These findings suggest that high cardiovascular intensity itself might, in part, contribute to a deleterious effect of exercise on bone.

Consistent with this idea, in well-trained triathletes, β-CTX concentrations are increased by 50% after 60 min of stationary cycling at 80% VO_{2max} whereas bone ALP concentrations remain unchanged (Guillemant *et al.*, 2004). As cycling imposes negligible mechanical loading due to the effects of gravity, and this population are well accustomed to this type of exercise, this finding might suggest that a high cardiovascular strain might increase bone resorption but not formation, resulting in a negative bone remodelling balance, at least in the short term.

Few studies have attempted to directly compare variations in exercise intensity in cardiovascular terms on bone turnover markers. These studies indicate that there might be a threshold above which bone markers (Herrmann *et al.*, 2007; Maimoun *et al.*, 2006), as well as parathyroid hormone (PTH) concentrations (Maimoun *et al.*, 2006) are stimulated. However, both studies used cycling as the mode of exercise and examined only two different exercise intensities. Additionally, one study took no samples for analysis beyond 15 min post-exercise (Maimoun *et al.*, 2006) while the other failed to standardise the time of day of the trials and the nutritional status of subjects (Herrmann *et al.*, 2007),

both of which are known to affect bone turnover markers, particularly β-CTX (Wichers *et al.*, 1999; Clowes *et al.*, 2002a; Henriksen *et al.*, 2003).

The aim of the present study, therefore, was to compare the effects of three different cardiovascular exercise intensities on changes in bone turnover markers during and for four days following acute, endurance running under highly standardised conditions.

6.2 Materials and Methods

6.2.1 Subjects

Ten men were recruited to participate in the study (Table 6.1). All subjects were in good physical condition, with a history of weight-bearing exercise and average to above average levels of fitness. The group was a mixture of team sports players and recreational runners and included one 'club' level runner. All subjects performed at least bout of endurance running per week. The study had a within-subject design with all subjects completing all experimental conditions.

6.2.2 Overview of exercise intervention

On Day 4, all subjects performed one, 60 min bout of treadmill running at 55% (LOW), 65% (MOD) or 75% (HIGH) of VO_{2max}, followed by 3 h of recovery. The order in which subjects completed the three conditions was counterbalanced.

6.2.3 Preliminary measures

Medical Screening: Subjects underwent a full medical examination as described in Section 3.2.1.

Dietary Analysis: Subjects completed a three-day food diary as described in Section 3.2.2.

Determination of the association between oxygen uptake and running velocity during level (0° gradient) treadmill running and of maximal rate of oxygen uptake: The association between oxygen uptake and running velocity during level running was determined as described in Section 3.2.3. Maximal rate of oxygen uptake was determined as described in Section 3.2.4. The results of the two tests were used to estimate the treadmill velocity corresponding to 50%, 55% 65% and 75% VO_{2max} during level running based on the regression line of VO_2 and treadmill velocity.

Experimental dietary provision: In Study II, due to difficulties in Study I in designing diets that contained 8 g CHO·kg FFM^{-1} but remained isocaloric with subjects' habitual diet, diets were designed for each subject consisting of 6 g CHO·kg FFM^{-1}. Despite this alteration, some experimental diets in Study II still contained a high CHO content, particularly if subjects did not have a high habitual energy intake. Thus, in this study it was decided to design a balanced diet for each subject consisting of approximately 55% carbohydrate (CHO), 30% fat and 15% protein (PRO). Subjects were provided with three menus that were given in a three-day cyclic order with Menu A on Days 1 and 5, Menu B on Days 2 and 6, and Menu C on Days 3 and 7 (for details of diet on Day 4 see *Section 6.2.4*). Details of menus for individual subjects can be found in Appendix A.

6.2.4 Trial Procedures

Day 4: Following an overnight fast, subjects arrived at the laboratory at 0730 h, provided a second void urine sample and had nude body mass measured (Mettler-Toledo ID7, Mettler-Toledo, Germany). Subjects subsequently adopted a semi-recumbent position on a bed and had a cannula (18GA 1.2x45 mm, Becton Dickinson, USA) inserted into a prominent forearm vein, where it remained until the final blood sample was collected. The cannula was kept patent with an isotonic saline solution (0.9% NaCl).

A baseline blood sample was collected at 0800 h for measurement of all biochemical markers and exercise commenced at 0815 h. Exercise bouts consisted of 60 min of treadmill running preceded by a 5 min warm-up at 50% VO_{2max}, separated by 5 min for volitional stretching.

On completion of exercise, subjects dried off, had their nude body mass measured, and rested in a semi-recumbent position for a further 3 h. The difference between pre- and post-exercise body mass was calculated (0.72 ± 0.27 kg, 0.80 ± 0.16 kg, 0.93 ± 0.24 kg in LOW, MOD and HIGH) and subjects consumed 1.5 ml of plain water for every gram change in body mass during the recovery period. Blood samples were collected at identical time points in the three conditions; at 0800 h (BASE), after 20 min, 40 min and 60 min of exercise (EX20 - EX60) and 0.5 h, 1.0 h, 2.0 h and 3.0 h of recovery (R0.5 - R3.0) (Figure 6.1).

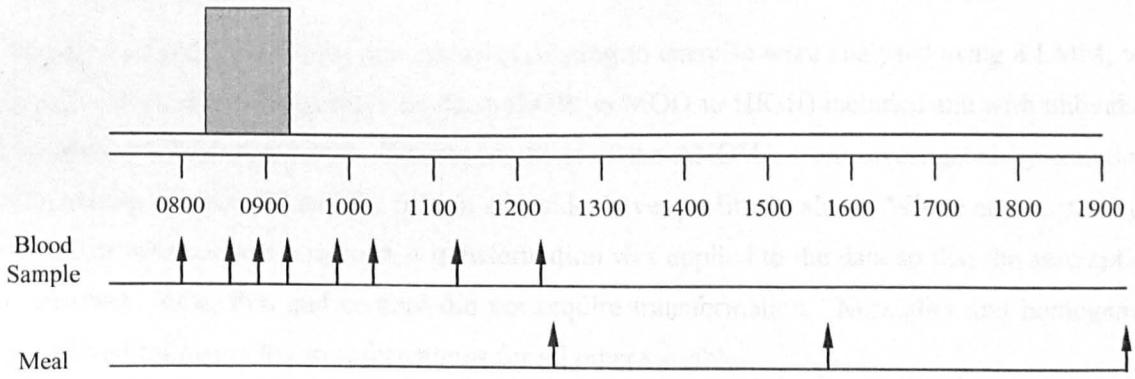

Figure 6.1. Experimental protocol on Day 4. Grey box indicates 60 min of treadmill exercise at 55% (LOW), 65% (MOD) or 75% (HIGH) VO_{2max}. Arrows indicate timing of blood samples and standardised meals. Subjects remained fasting until the blood sample at 3 h post-exercise (R3.0) was collected.

Running speeds in the LOW, MOD and HIGH conditions were 8.5 ± 1.0, 10.2 ± 1.1 and 11.9 ± 1.2 $km \cdot h^{-1}$. Sixty second samples of expired air (inspiration to inspiration) were collected and analysed as described in Section 3.3.1. Ratings of perceived exertion (RPE) (Noble *et al.*, 1983) were recorded immediately before each expired air sample. Heart rate was recorded continuously and water was consumed *ad libitum* (0.27 ± 0.14 L, 0.28 ± 0.10 L, and 0.34 ± 0.18 L in LOW, MOD and HIGH) during exercise. The ambient room temperature during exercise was 20 ± 1 °C in all conditions.

Subjects consumed a standardised diet (13.2 MJ, 53% CHO, 32% FAT, 15 % PRO, 767 mg calcium) divided into three meals. The first meal was eaten in the laboratory immediately following the final blood sample at 3 h post-exercise. The further two meals were taken home by subjects to be consumed around 1600 h and 1930 h (Figure 6.1). Only plain water was allowed after 2100 h. The same diet was provided in all three conditions.

6.2.5 Biochemical analysis

All blood samples were analysed for glucose, lactate, cortisol, β-CTX, P1NP, OPG, PTH, ACa and PO_4. Bone ALP was measured in samples taken at BASE and on the four follow-up days (FU1 – FU4) only. All urine samples were analysed for fPYD, fDPD and Cr and the volume of each sample was also recorded. Pyridinoline concentrations were expressed as a ratio with Cr concentrations. Pyridinoline 'output' (in nmol) was also calculated by multiplying pyridinoline concentrations ($nmol \cdot L^{-1}$) by urine volume (L).

6.2.6 Statistical Analysis

All data are presented as mean ± 1SD unless otherwise stated. Statistical significance was accepted at an alpha level of $P < 0.05$. A one-way ANOVA was used to compare baseline biochemistry in the LOW, MOD and HIGH groups. Paired sample t-tests were used to compare habitual with experimental dietary data.

All biochemical data, body mass and variables relating to exercise were analysed using a LMM, with the factors *Time* (of sampling) and *Condition* (LOW *vs* MOD *vs* HIGH) included and with individuals as a random within-group factor. The assumptions of the ANOVA were investigated by examining the distribution of residuals and the pattern of residuals versus fitted values. Where non-normality or non-constant variance was observed, a transformation was applied to the data so that the assumptions were satisfied. ACa, PO_4 and cortisol did not require transformation. Normality and homogeneity were achieved following log transformations for all other variables.

Where there was a significant main effect of *Time* but no significant *Condition* x *Time* interaction, each subsequent time point was compared against BASE using a pooled mean using Dunnett's test with BASE as the '*Control*'. When the *Condition* x *Time* interaction was significant, within each group each subsequent time point was compared against BASE using Dunnett's test with BASE as the '*Control*' and groups were compared to each other at all time points using the SNK test.

Pearson's correlation coefficient was calculated between the percentage changes in PO_4 and PTH from BASE to EX20 for the LOW, MOD and HIGH groups.

6.3 Results

6.3.1 Subject characteristics

Subject characteristics are shown in Table 6.1.

Table 6.1. Subject characteristics

Measure	Value
n	10
Age (y)	28 ± 4
Body Mass (kg)	75.8 ± 6.4
Height (m)	1.81 ± 0.07
BMI ($kg \cdot m^{-2}$)	23.2 ± 2.1
Body fat (%)	15.5 ± 6.0
VO_{2max} ($ml \cdot kg^{-1} \cdot min^{-1}$)	56 ± 8

Values are mean ± 1SD. BMI, body mass index.

6.3.2 Dietary analysis and experimental dietary provision

Dietary analysis: Energy intake, macronutrient composition and dietary calcium from food diary analysis are shown in Table 6.2.

Table 6.2. Habitual energy intake, macronutrient composition and dietary calcium content.

Measure	Value
Energy (MJ)	10.9 ± 1.9
CHO (g)	361.6 ± 71.7
CHO ($g \cdot kg\ FFM^{-1}$)	5.5 ± 1.3
CHO (% of total energy)	52.4 ± 8.2
Fat (% of total energy)	29.3 ± 7.4
Protein (% of total energy)	16.9 ± 2.8
Calcium ($mg \cdot day^{-1}$)	1160 (range, 724 - 2035)

Values are mean ± 1SD unless otherwise stated. CHO, carbohydrate; FFM, fat-free mass.

Experimental dietary provision: The energy content and macronutrient composition of the experimental diets are shown in Table 6.3. There were no significant differences for any dietary variable between reported habitual intake and experimental diets.

Table 6.3. Energy content and macronutrient composition of the experimental diets consumed by subjects on Days 1-3 and 5-7 of each experimental condition.

Measure	Value
Energy (MJ)	11.0 ± 1.7
CHO (g)	380.9 ± 65.4
CHO (g·kg FFM^{-1})	6.0 ± 1.1
CHO (% of total energy)	54.6 ± 2.5
FAT (% of total energy)	29.8 ± 1.9
PRO (% of total energy)	15.6 ± 0.9
Calcium (mg·day^{-1})	1125 (range, 770 – 1490)

Values are mean ± 1SD unless otherwise stated. CHO, carbohydrate; FFM, fat-free mass.

6.3.3 Baseline biochemistry

There were no significant differences in baseline biochemistry between the three groups (Table 6.4).

Table 6.4. Baseline biochemistry in the LOW, MOD and HIGH groups.

Marker	LOW	MOD	HIGH	P Value (one-way ANOVA)
β-CTX (ug·L^{-1})	0.71 ± 0.22	0.69 ± 0.25	0.69 ± 0.22	0.964
P1NP (ug·L^{-1})	55 ± 26	56 ± 28	56 ± 22	0.998
OC (ug·L^{-1})	28.9 ± 9.3	28.2 ± 8.3	28.5 ± 7.8	0.983
bone ALP (U·L^{-1})	29 ± 10	28 ± 11	27 ± 9	0.968
Cr (umol·L^{-1})	16.8 ± 7.0	15.3 ± 6.6	15.0 ± 6.2	0.812
fPYD/Cr (nmol·mmol Cr^{-1})	15.6 ± 2.0	15.4 ± 2.1	14.9 ± 2.3	0.743
fDPD/Cr (nmol·mmol Cr^{-1})	4.4 ± 0.6	4.3 ± 0.6	4.0 ± 0.7	0.339
OPG (pmol·L^{-1})	1.7 ± 0.6	1.8 ± 0.5	1.8 ± 0.6	0.922
PTH (pmol·L^{-1})	4.2 ± 1.5	4.4 ± 1.3	4.8 ± 1.7	0.666
ACa (mmol·L^{-1})	2.34 ± 0.05	2.33 ± 0.07	2.33 ± 0.06	0.896
PO_4 (mmol·L^{-1})	1.16 ± 0.13	1.15 ± 0.16	1.17 ± 0.14	0.972
Cortisol (nmol·L^{-1})	424 ± 101	464 ± 116	465 ± 96	0.618

Values are mean ± 1SD unless otherwise stated.

6.3.4 Body mass

There was a significant main effect of *Time* ($P < 0.05$) but no significant *Condition* x *Time* interaction ($P = 0.970$) for body mass measured from Day 4 (BASE) to Day 8 (FU4). Pooled, mean data from the three groups showed a small (0.5%) but significant ($P < 0.05$) decrease in body mass from BASE at FU4 (Figure 6.2).

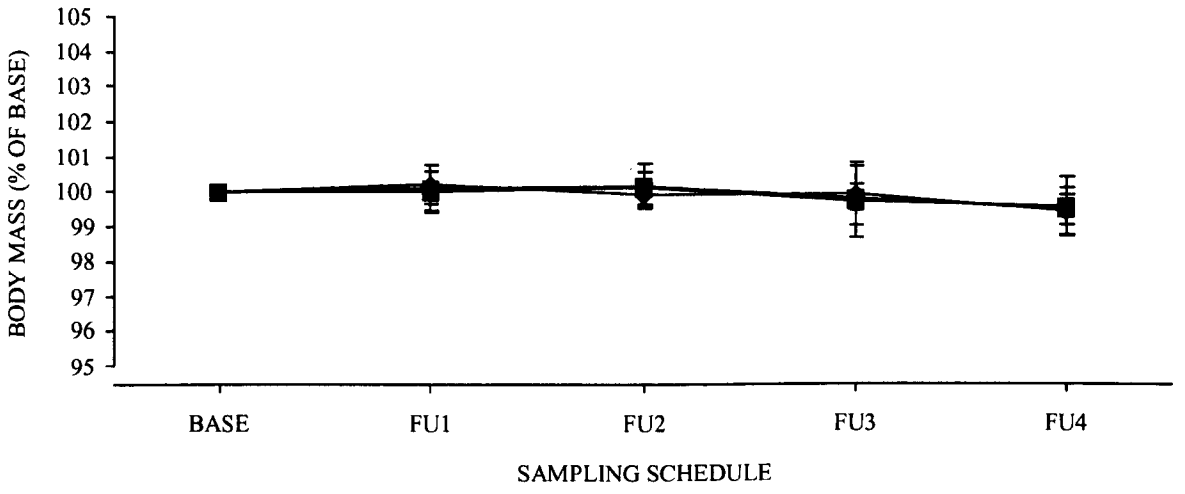

Figure 6.2. Percentage change in body mass from baseline (BASE) on the four follow-up days (FU1 – FU4) in the LOW (filled diamonds), MOD (open squares) and HIGH (filled squares) conditions. Values are mean ± 1SD. Pooled, body mass was significantly lower than BASE at FU4 only.

6.3.5 Exercise variables

Measured exercise intensities were 55 ± 3%, 63 ± 3% and 75 ± 3% VO_{2max} in the LOW, MOD and HIGH conditions. Oxygen uptake (VO_2), heart rate, RER and RPE all increased significantly ($P < 0.001$) with increasing exercise intensity (Table 6.5).

Table 6.5. Oxygen uptake (VO_2), heart rate (% of max HR) respiratory exchange ratio (RER) and ratings of perceived exertion (RPE) during 60 min of exercise at 55% VO_{2max} (LOW), 65% VO_{2max} (MOD) and 75% VO_{2max} (HIGH).

Variable	LOW	MOD	HIGH	CONDITION
Treadmill speed ($km \cdot h^{-1}$)	8.5 ± 1.0	10.2 ± 1.1 [i]	11.9 ± 1.2 [c,f]	P < 0.001
VO_2 ($L \cdot min^{-1}$)	2.3 ± 0.3	2.6 ± 0.3 [i]	3.2 ± 0.3 [c,f]	P < 0.001
HR (% of HR_{max})	75 ± 7	82 ± 4 [i]	92 ± 4 [c,f]	P < 0.001
RER	0.855 ± 0.041	0.867 ± 0.048	0.898 ± 0.032 [c,e]	P < 0.001
RPE	10 ± 1	12 ± 2 [i]	14 ± 2 [c,f]	P < 0.001

Values are mean ± 1SD. [c] different ($P < 0.001$) from LOW; [e] different ($P < 0.01$) from MOD; [f] different ($P < 0.001$) from MOD; [i] different ($P < 0.001$) from HIGH.

6.3.6 Glucose, lactate and cortisol

Glucose: There was a significant main effect of *Time* ($P < 0.001$) but no significant *Group* x *Time* interaction ($P = 0.449$) for glucose concentrations. Pooled, mean concentrations were significantly increased ($P < 0.001$) from BASE throughout exercise (Figure 6.3, Panel A). Concentrations increased from approximately 4.3 $mmol \cdot L^{-1}$ at BASE to 4.5 $mmol \cdot L^{-1}$ at FD60 in LOW and MOD and 5.3 ± 0.8 $mmol \cdot L^{-1}$ in HIGH. Following exercise, glucose concentrations were reduced from BASE at R1.0 ($P < 0.001$) and R3.0 ($P < 0.01$).

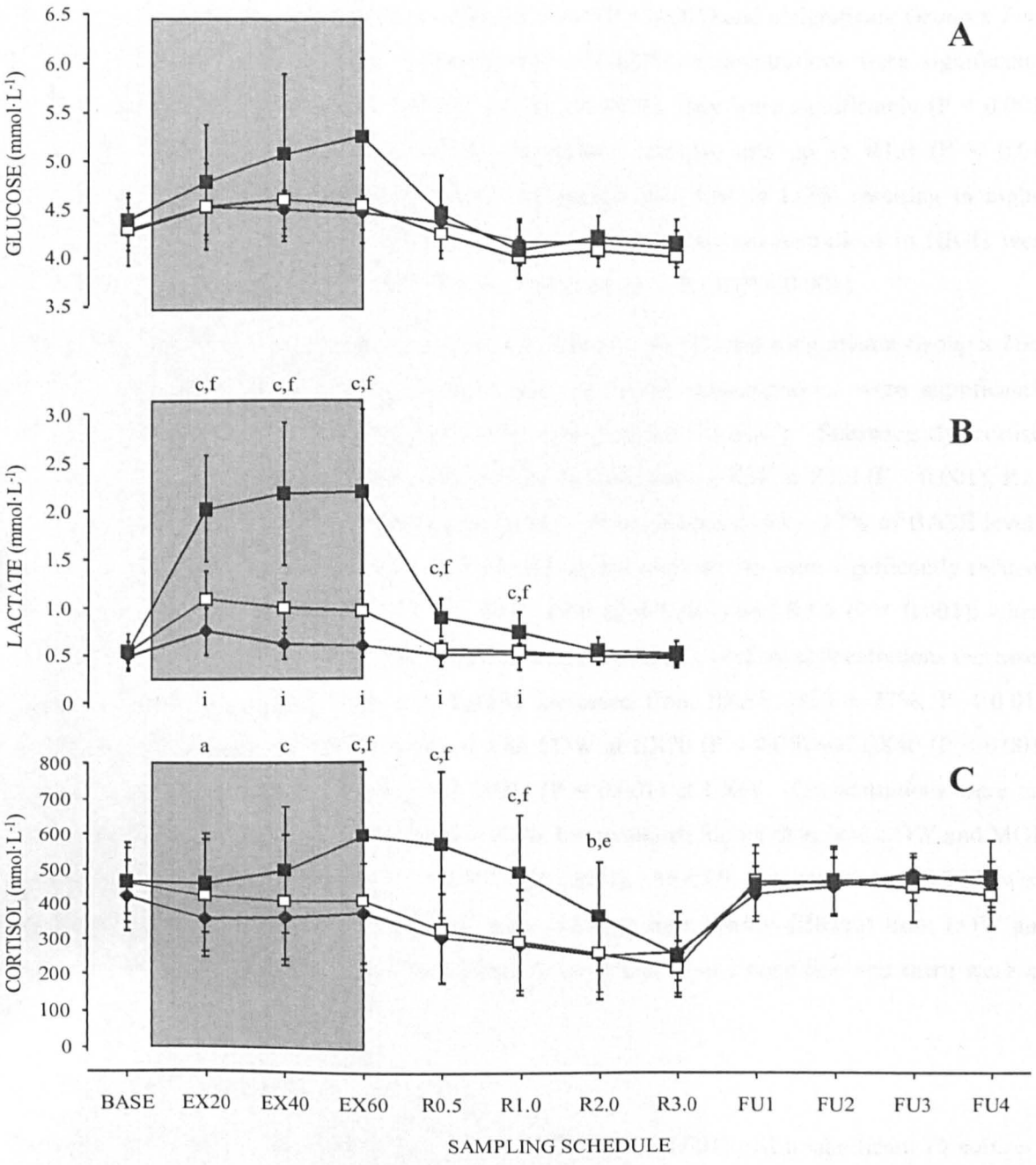

Figure 6.3. Glucose (Panel A), lactate (Panel B) and cortisol (Panel C) concentrations at baseline (BASE), during 60 min of exercise (EX20 – EX60), during 3 h of recovery (R0.5 – R3.0) and on 4 follow up days (FU1 – FU4) in the LOW (filled diamonds), MOD (open squares) and HIGH (filled squares) conditions. Values are mean ± 1SD. Pooled, glucose concentrations were significantly higher than BASE throughout exercise and significantly lower than BASE at R1.0 and R3.0. Lactate concentrations were significantly higher than BASE at EX20 only in LOW, throughout exercise in MOD and throughout exercise and up to R1.0 in HIGH. Cortisol concentrations were significantly lower than BASE from EX20 inwards in LOW and significantly lower from R0.5 to R3.0 only in MOD. In HIGH, concentrations were significantly higher than BASE at EX60 and significantly lower at R3.0. [a] HIGH different ($P < 0.05$) from LOW; [b] HIGH different ($P < 0.01$) from LOW; [c] HIGH different ($P < 0.001$) from LOW; [e] HIGH different ($P < 0.01$) from MOD; [f] HIGH different ($P < 0.001$) from MOD; [i] MOD different ($P < 0.001$) from LOW.

Lactate: There was both a significant main effect of *Time* ($P < 0.001$) and a significant *Group* x *Time* interaction ($P < 0.001$) for glucose concentrations. IN LOW, concentrations were significantly ($P < 0.001$) increased from BASE in LOW at EX20. In MOD, they were significantly ($P < 0.001$) increased throughout exercise, and in HIGH, throughout exercise and up to R1.0 ($P < 0.01$) (Figure 6.3, Panel B). The increase in MOD was greater than that in LOW resulting in higher concentrations at EX20, EX40 and EX60 ($P < 0.001$), while lactate concentrations in HIGH were higher than in both LOW and MOD throughout exercise and up to R1.0 ($P < 0.001$).

Cortisol: There was both a significant main effect of *Time* ($P < 0.001$) and a significant *Group* x *Time* interaction ($P < 0.01$) for cortisol concentrations. In LOW, concentrations were significantly ($P < 0.05$) reduced from BASE throughout exercise (Figure 6.3, Panel C). Subsequently, cortisol concentrations declined further and were significantly reduced from BASE at R1.0 ($P < 0.001$), R2.0 ($P < 0.001$) and R3.0 ($P < 0.001$) where concentrations were reduced to 65 ± 17% of BASE levels. In MOD, concentrations were unchanged from BASE during exercise but were significantly reduced from BASE at R0.5 ($P < 0.05$), R1.0 ($P < 0.001$), R2.0 ($P < 0.001$) and R3.0 ($P < 0.001$), where concentrations were reduced to 51 ± 18% of BASE levels. In HIGH, cortisol concentrations increased during exercise and, by EX60, were significantly increased from BASE (+30 ± 37%; $P < 0.01$), resulting in higher cortisol in HIGH compared with LOW at EX20 ($P < 0.05$) and EX40 ($P < 0.001$) and higher than both LOW ($P < 0.001$) and MOD ($P < 0.001$) at EX60. Concentrations were not significantly different from BASE from R0.5 to R2.0, but remained higher than both LOW and MOD at R0.5 ($P < 0.001$) and R1.0 ($P < 0.001$) and R2.0 ($P < 0.01$). At R3.0, concentrations in HIGH were significantly reduced ($P < 0.001$) from BASE and no longer significantly different from LOW and MOD. At FU1, concentrations were not different from BASE in any condition and there were no further changes in cortisol thereafter.

6.3.7 Bone turnover markers

β-CTX: There was both a significant main effect of *Time* ($P < 0.001$) and a significant *Condition* x *Time* interaction ($P < 0.05$) for β-CTX concentrations. In LOW, β-CTX concentrations declined by 15% during exercise and were significantly lower than BASE at EX60 ($P < 0.05$) (Figure 6.4, Panel A). β-CTX remained significantly lower than BASE at R0.5 ($P < 0.01$), R1.0 ($P < 0.05$), R2.0 ($P < 0.001$) and R3.0 ($P < 0.001$). In MOD, β-CTX concentrations declined by 10% during exercise, although this difference did not reach the assigned level of significance. β-CTX concentrations reduced further during recovery and were significantly reduced from BASE at R2.0 ($P < 0.001$) and R3.0 ($P < 0.001$). In HIGH, β-CTX concentrations were not significantly different from BASE during exercise and were significantly higher than LOW (+18%, $P < 0.01$) and MOD (+13%, $P < 0.05$) at EX60.

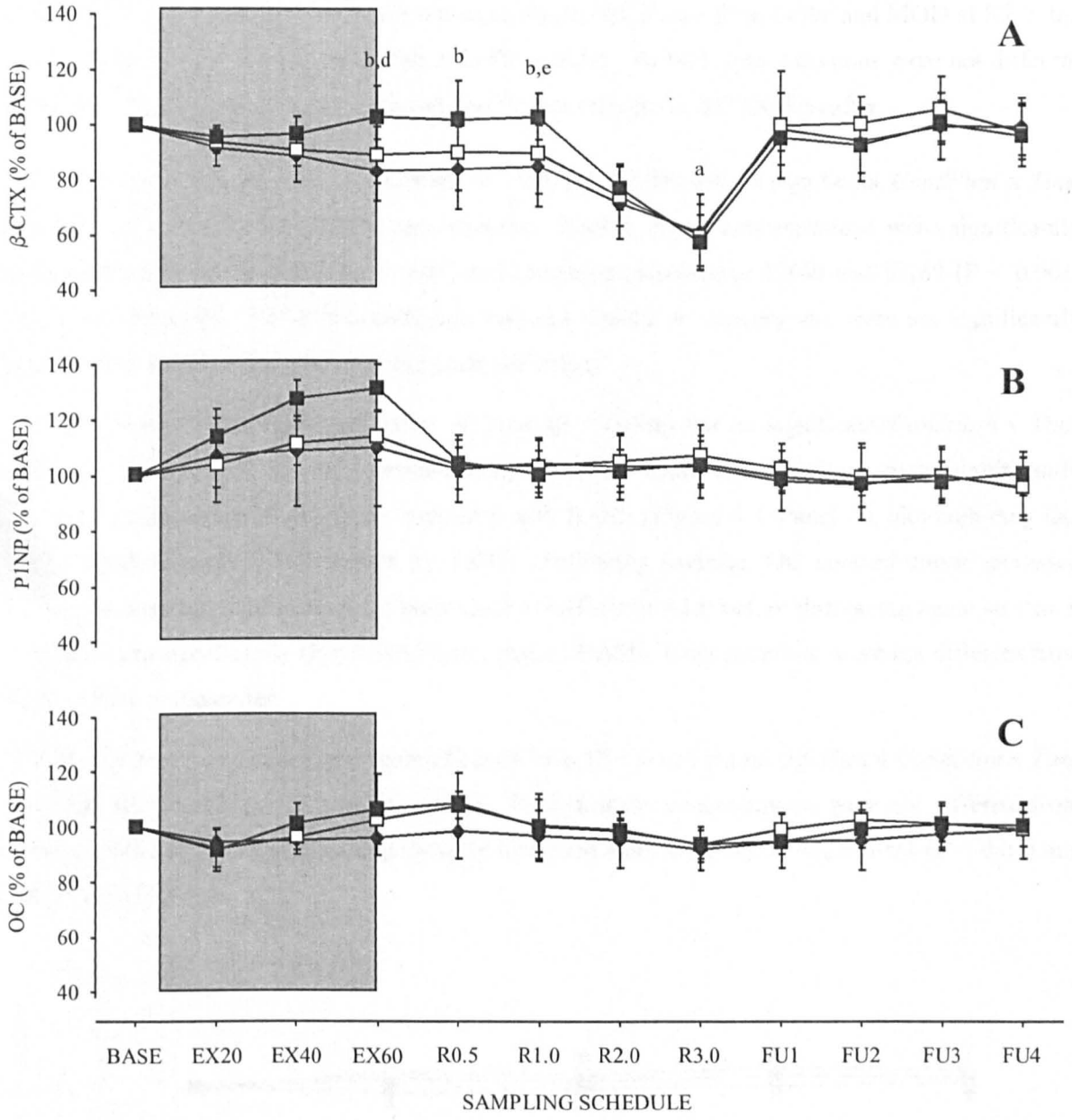

Figure 6.4. β-CTX (Panel A), P1NP (Panel B) and OC (Panel C) concentrations, expressed as a percentage of baseline (BASE) values, during 60 min of exercise (EX20 – EX60), 3 h of recovery (R0.5 – R3.0) and on 4 follow up days (FU1 – FU4) in the LOW (filled diamonds), MOD (open squares) and HIGH (filled squares) conditions. Values are mean ± 1SD. β-CTX concentrations in LOW were significantly lower than BASE at EX60 and up to R3.0, and from R1.0 to R3.0 only in both MOD and HIGH. Pooled, P1NP concentrations were significantly higher than BASE at EX20 and up to EX60. Pooled, OC concentrations were significantly lower than BASE at EX20 and again at R3.0. [a] HIGH different ($P < 0.05$) from LOW; [b] HIGH different ($P < 0.01$) from LOW; [d] HIGH different ($P < 0.01$) from MOD; [e] HIGH different ($P < 0.01$) from MOD.

The magnitude of this difference was maintained in the first hour post-exercise, with concentrations in HIGH significantly higher than LOW at R0.5 ($P < 0.01$), and higher than both LOW and MOD at R1.0 ($P < 0.01$). Subsequently, concentrations in HIGH decreased and were significantly lower ($P < 0.001$)

than BASE at R2.0 and R3.0 and were not significantly difference from LOW and MOD at R2.0, but were significantly lower ($P < 0.05$) than in LOW at R3.0. At FU1, concentrations were not different from BASE in any condition and there were no further changes in β-CTX thereafter.

P1NP: There was a significant main effect of *Time* ($P < 0.05$) but no significant *Condition* x *Time* interaction ($P = 0.477$) for P1NP concentrations. Pooled, mean concentrations were significantly increased from BASE at EX20 ($P < 0.01$) and remained increased at EX40 and EX60 ($P < 0.001$) (Figure 6.4, Panel B). P1NP concentrations reduced rapidly in recovery and were not significantly different from BASE at R0.5 or any time point thereafter.

OC: There was a significant main effect of *Time* ($P < 0.001$) but no significant *Condition* x *Time* interaction ($P = 0.515$) for OC concentrations. Pooled, mean concentrations were significantly ($P < 0.001$) reduced by 5% at EX20 compared with BASE (Figure 6.4, Panel C), although they had returned back towards BASE values by EX40. Following exercise, OC concentrations increased slightly but were not significantly different from BASE up to R1.0 before decreasing again so that at R3.0 they were significantly ($P < 0.001$) lower than at BASE. Concentrations were not different from BASE at FU1 or thereafter.

Bone ALP: There was a significant main effect of *Time* ($P < 0.05$) but no significant *Condition* x *Time* interaction ($P = 0.722$) for OC concentrations. Pooled, mean concentrations were not different from BASE at FU1 and FU2 but were significantly increased from baseline by 4% at FU3 ($P < 0.01$) and FU4 ($P < 0.01$) (Figure 6.5).

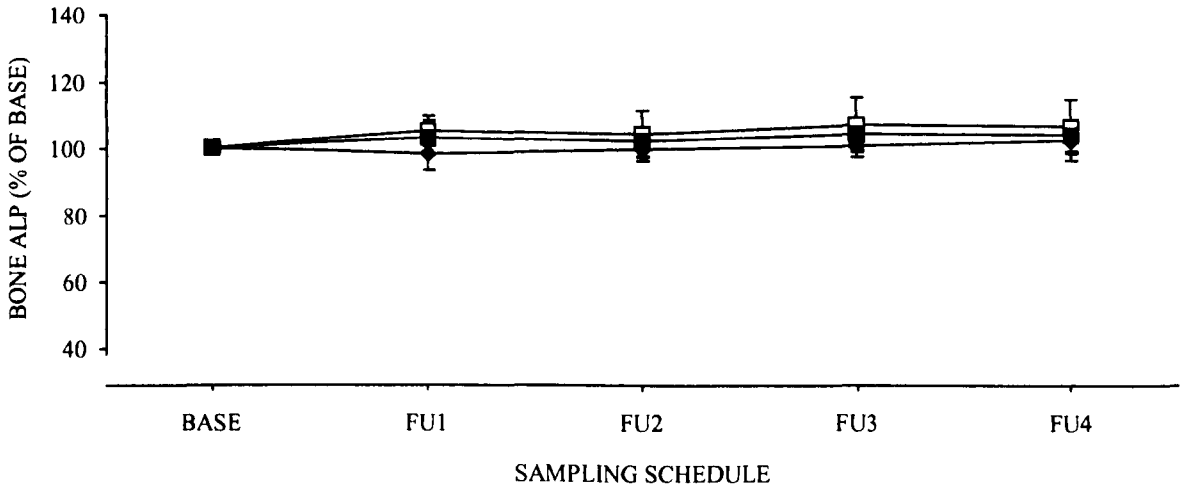

Figure 6.5. Bone ALP concentrations, expressed as a percentage of baseline (BASE) values on 4 follow up days (FU1 – FU4) in the LOW (filled diamonds), MOD (open squares) and HIGH (filled squares) conditions. Values are mean ± 1SD. Pooled, bone ALP concentrations were significantly higher than BASE at FU3 and FU4.

fPYD/Cr: There was a significant main effect of *Time* ($P < 0.05$) but no significant *Condition* x *Time* interaction ($P = 0.557$) for fPYD/Cr concentrations. Mean, pooled concentrations tended to be higher than BASE at FU3 ($P = 0.07$) but no individual time points were significantly different from BASE (Figure 6.6, Panel A).

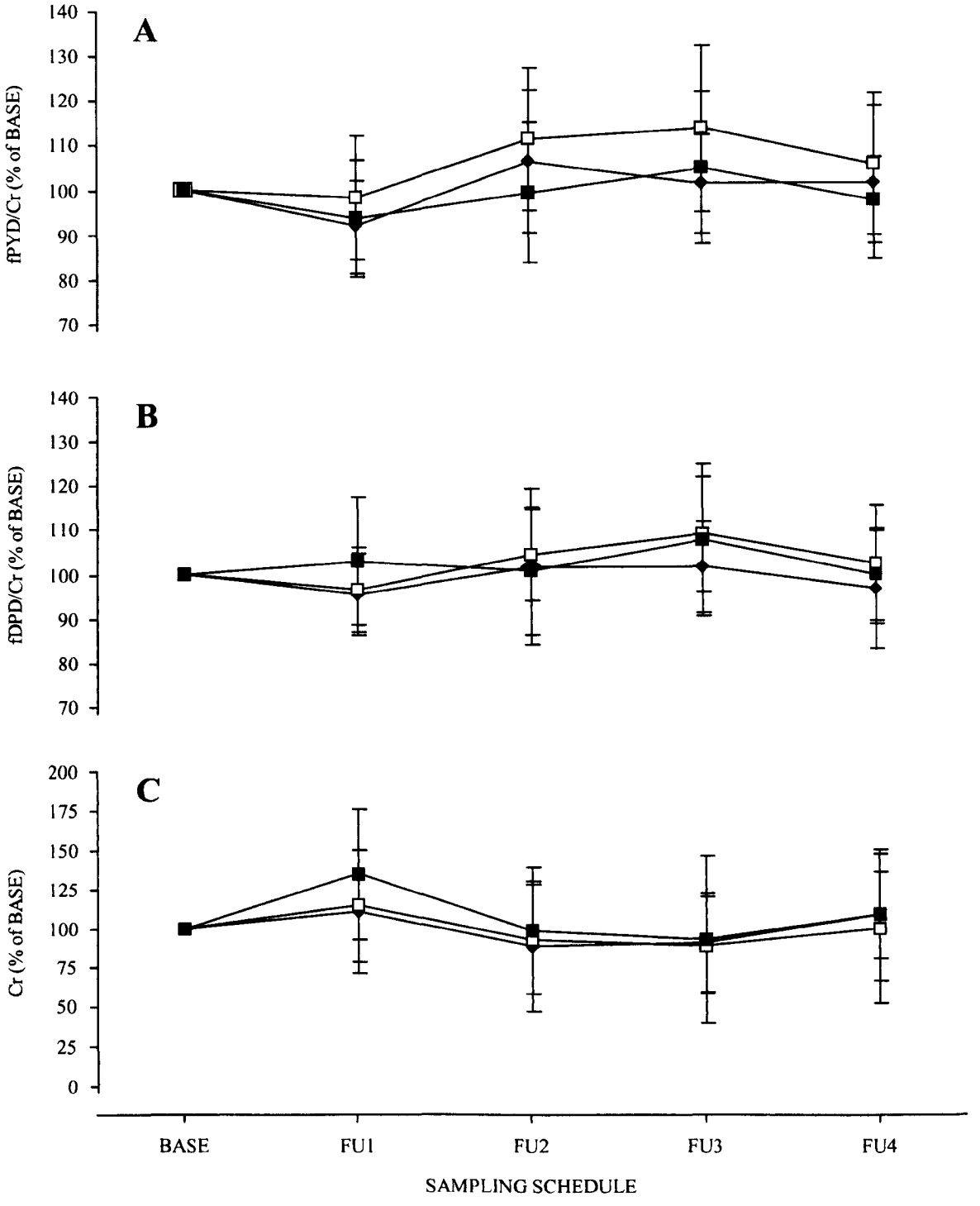

Figure 6.6. The percentage change in BASE concentrations of fPYD/Cr (Panel A), fDPD/Cr (Panel B) and Cr (Panel C) on the four recovery days (FU1 – FU4) in the LOW (filled diamonds), MOD (open squares) and HIGH (filled squares) conditions. Values are mean ± 1SD.

fDPD/Cr: There was a significant main effect of *Time* ($P < 0.05$) but no significant *Condition* x *Time* interaction ($P = 0.619$) for DPD/Cr concentrations. Mean, pooled concentrations tended to be higher than BASE at FU3 ($P = 0.09$) but no individual time points were significantly different from BASE (Figure 6.6, Panel B).

Cr: There was a significant main effect of *Time* ($P < 0.01$) but no significant *Condition* x *Time* interaction ($P = 0.881$) for Cr concentrations. Mean, pooled concentrations tended to be lower than BASE at FU3 ($P = 0.08$) but no individual time points were significantly different from BASE (Figure 6.6, Panel C). There was considerable variation in creatinine levels both within and between individual subjects. At FU1, Cr levels varied from 61 to 221% of BASE values with levels between 33% and 200% at FU2 to FU4. Several subjects showed variations in Cr levels of more than 30% above and below BASE values on consecutive days (Figure 6.7, Panels A, B and C).

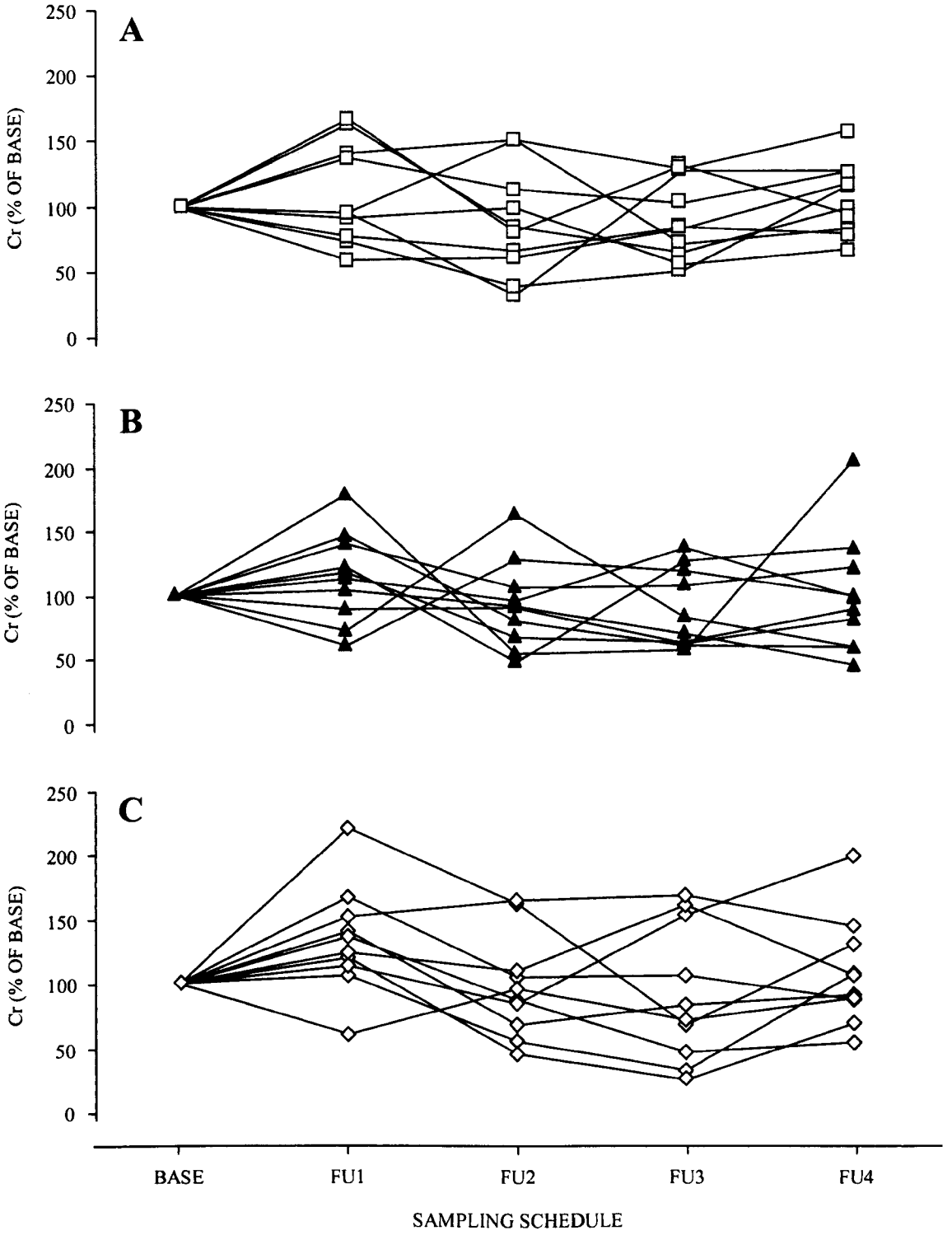

Figure 6.7. Individual changes in Cr, expressed as the percentage change in baseline (BASE) concentrations on the four follow-up days (FU1 – FU4) in the LOW (Panel A), MOD (Panel B) and HIGH (Panel C) conditions.

fPYD output: There was no significant main effect of *Time* (P = 0.214) and no significant *Condition* x *Time* interaction (P = 0.340) for fPYD output (Figure 6.8, Panel A).

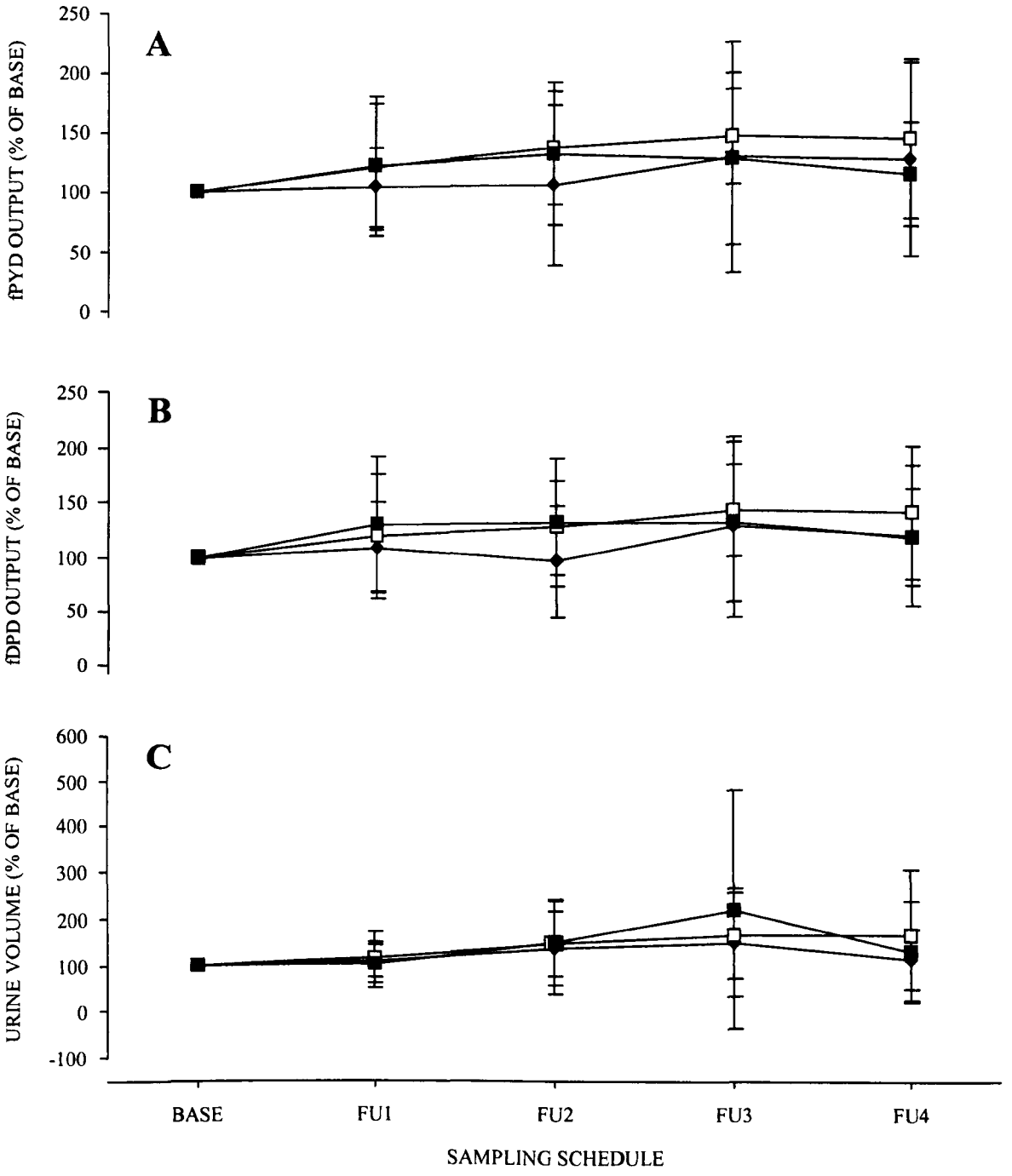

Figure 6.8. The percentage change from BASE of fPYD (Panel A) and fDPD (Panel B) output, and urine volume (Panel C), on the four recovery days (FU1 – FU4) in the LOW (filled diamonds), MOD (open squares) and HIGH (filled squares) conditions. Values are mean ± 1SD. fPYD or fDPD output (mmol) = fPYD or fDPD ($mmol \cdot L^{-1}$) x urine volume (L).

fDPD output: There was no significant main effect of *Time* (P = 0.171) and no significant *Condition* x *Time* interaction (P = 0.346) for fDPD output (Fig 6.8, Panel B).

Urine volume: There was tendency for increased urine volumes but the main effect of *Time* did not reach statistical significance (P = 0.058). There was also no significant *Condition* x *Time* interaction (P = 0.883) (Figure 6.8, Panel C). There was considerable variability in BASE urine volume between individual subjects (0.012 L to 0.176 L). This variability increased in samples across all five sampling points (0.012 L to 0.280 L). There was no consistent pattern of change from BASE across the four follow-up days with both increases and decreases in the volume of urine produced compared to that at BASE (Fig 6.9, Panels A, B and C). There was also considerable variability within individual subjects across the four follow-up days with decreases in the volume produced of up to 70% in several subjects, and increases in excess of 500% at some time points.

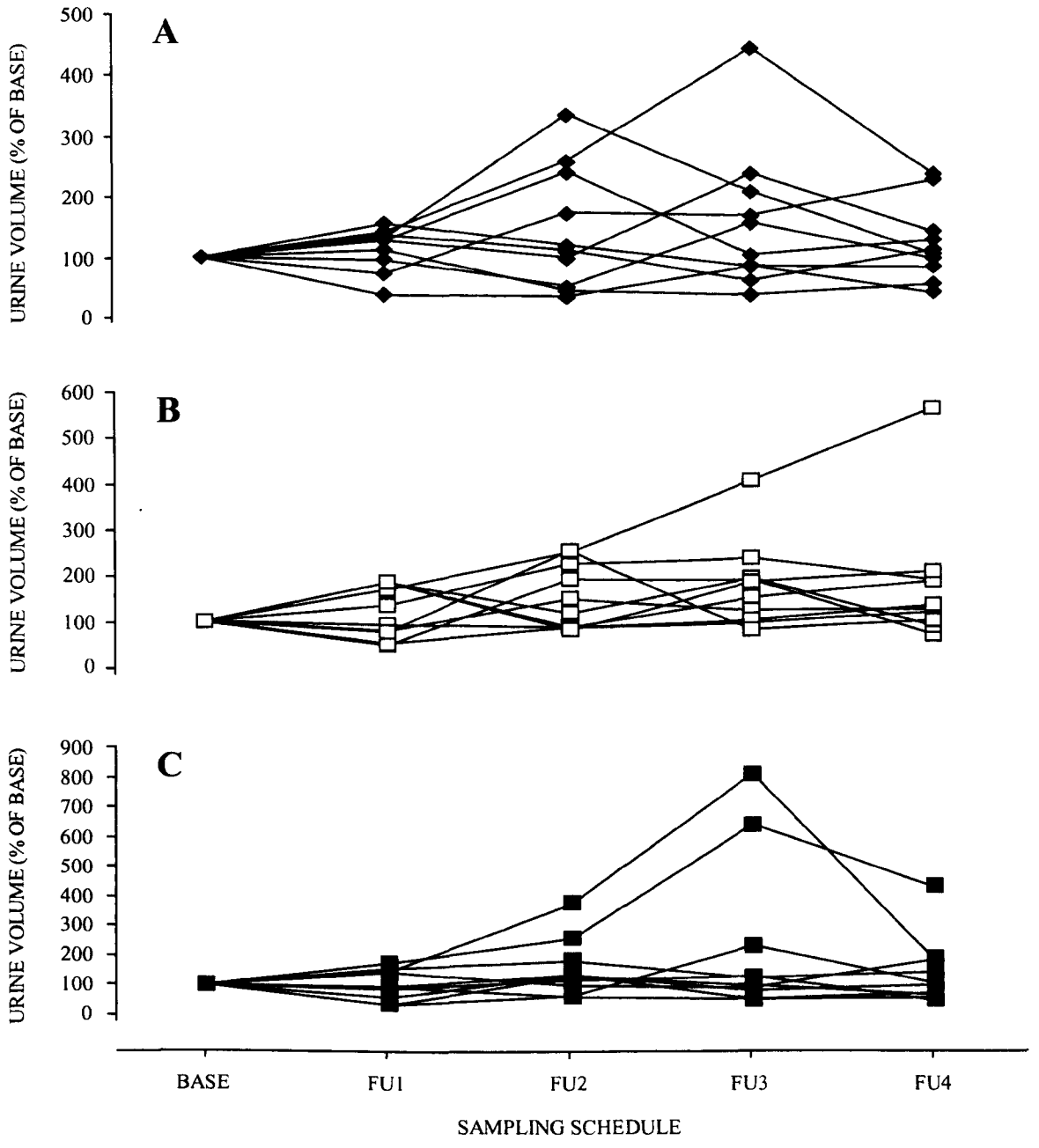

Figure 6.9. Individual changes in urine volume, expressed as the percentage change in baseline (BASE) concentrations on the four follow-up days (FU1 – FU4) in the LOW (Panel A), MOD (Panel B) and HIGH (Panel C) conditions.

6.3.8 OPG

There was a significant main effect of *Time* ($P < 0.001$) but no significant *Condition* x *Time* interaction ($P = 0.735$) for OPG concentrations. Pooled, mean concentrations were significantly increased ($P < 0.001$) at EX20 (LOW, 15 ± 25%; MOD, 25 ± 17%; HIGH, 41 ± 28%) and remained increased ($P < 0.001$) at EX60 (LOW, 25 ± 20%; MOD, 23 ± 25%; HIGH, 35 ± 28%) and during the first 3 h of recovery (Figure 6.10). Concentrations were not different from BASE at FU1 but showed a significant ($P < 0.01$) increase at FU3, but not FU4.

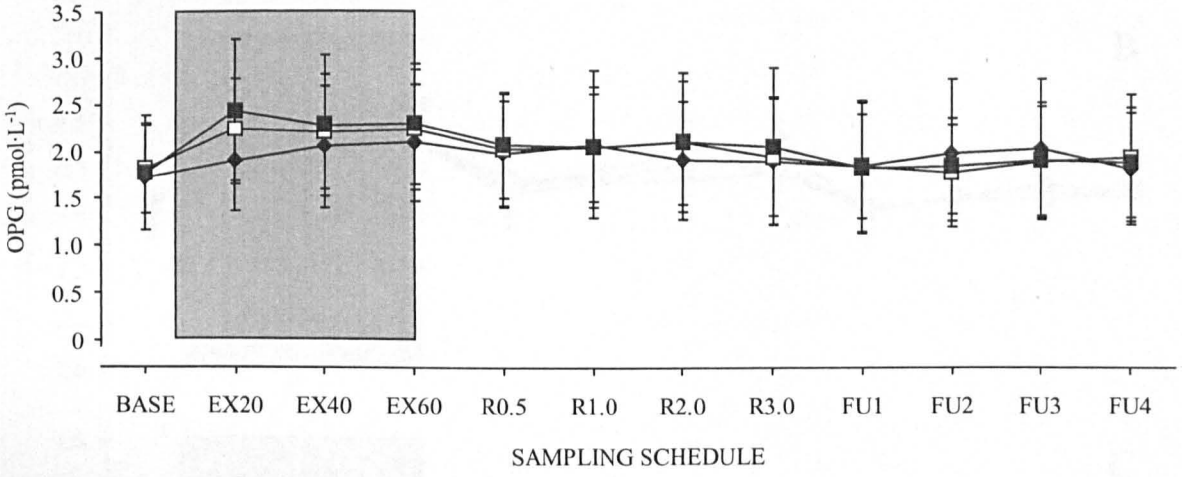

Figure 6.10. OPG concentrations at baseline (BASE), during 60 min of exercise (EX20 – EX60), during 3 h of recovery (R0.5 – R3.0) and on 4 follow up days (FU1 – FU4) in the LOW (filled diamonds), MOD (open squares) and HIGH (filled squares) conditions. Values are mean ± 1SD. Pooled, OPG concentrations were significantly higher than BASE throughout exercise and up to R3.0.

6.3.9 Calcium Metabolism

PTH: There was both a significant main effect of *Time* ($P < 0.001$) and a significant *Condition* x *Time* interaction ($P < 0.001$) for PTH concentrations. PTH was unchanged in LOW during exercise and recovery (Figure 6.11, Panel A). In MOD, PTH was unchanged during exercise but was significantly reduced ($P < 0.05$) from BASE by 19-21% from R1.0 to R3.0. In HIGH, there was a progressive increase in PTH during exercise. Concentrations were significantly increased from BASE at EX20 and EX40 ($P < 0.001$), and at EX20 were significantly higher than in LOW ($P < 0.05$), and higher than both LOW ($P < 0.001$) and MOD ($P < 0.001$) at EX40. Peak concentrations (9.0 ± 4.2 $pmol \cdot L^{-1}$) occurred at EX60, when concentrations were significantly increased ($P < 0.001$) 86 ± 47% from BASE and higher than both LOW and MOD ($P < 0.001$). PTH concentrations were not different from BASE or from LOW or MOD at R0.5, but were significantly reduced ($P < 0.001$) from BASE at R1.0 (15%), R2.0 (20%) and R3.0 (37%). Concentrations were not different from BASE at FU1 or thereafter in any condition.

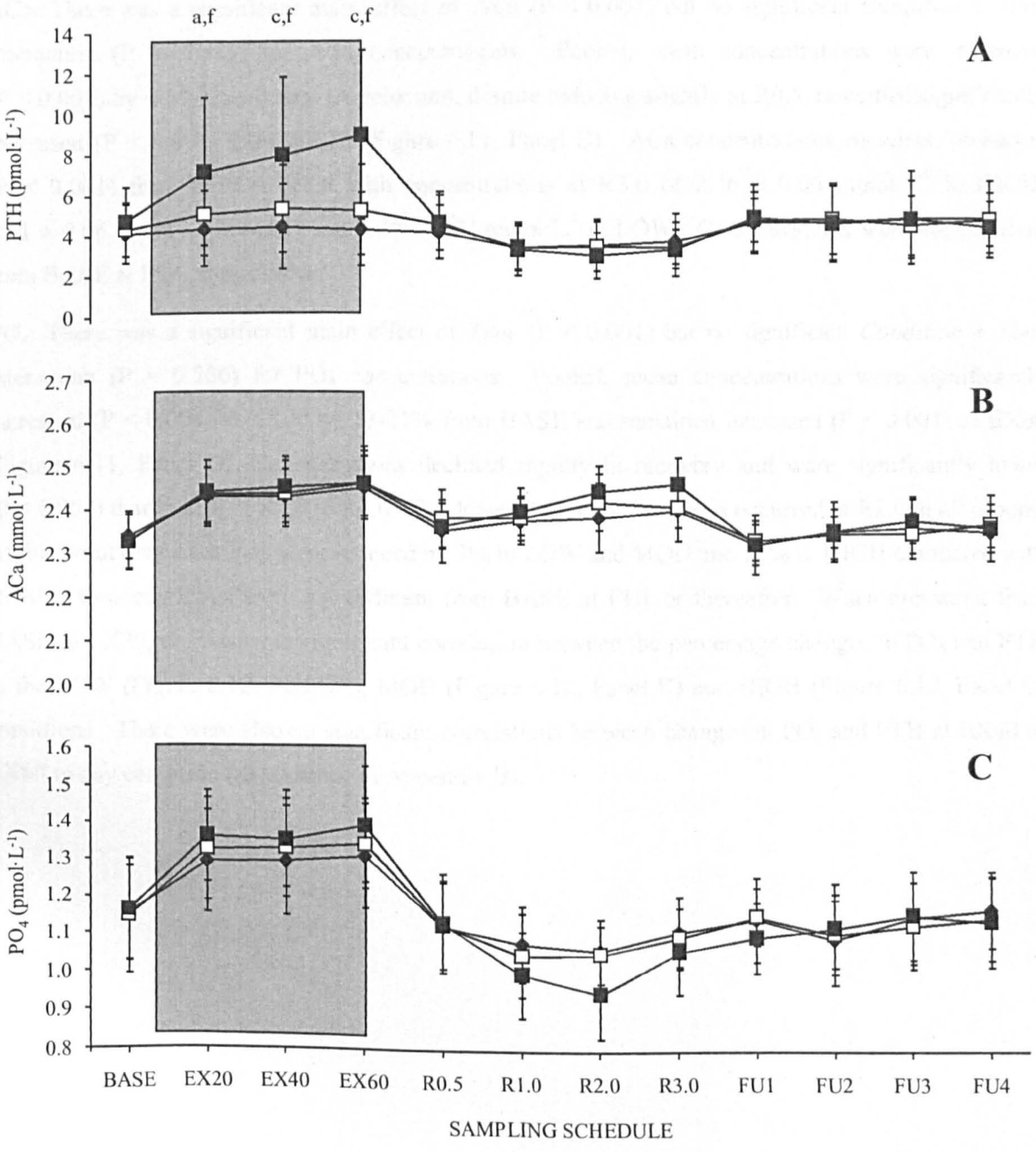

Figure 6.11. PTH (Panel A), ACa (Panel B) and PO_4 (Panel C) concentrations at baseline (BASE), during 60 min of exercise (EX20 – EX60), during 3 h of recovery (R0.5 – R3.0) and on 4 follow up days (FU1 – FU4) in the LOW (filled diamonds), MOD (open squares) and HIGH (filled squares) conditions. Values are mean ± 1SD. There was no significant change in PTH concentrations in LOW and in MOD, concentrations were significantly lower than BASE from R1.0 to R3.0 only. IN HIGH, PTH concentrations were significantly increased from BASE throughout exercise and significantly lower from R1.0 to R3.0. Pooled, ACa concentrations were higher than BASE throughout exercise and up to R3.0. Pooled, PO_4 concentrations were higher than BASE throughout exercise and significantly lower from R1.0 to R3.0. [a] HIGH different ($P < 0.05$) from LOW; [c] HIGH different ($P < 0.001$) from LOW; [f] HIGH different ($P < 0.001$) from MOD.

ACa: There was a significant main effect of *Time* ($P < 0.001$) but no significant *Condition* x *Time* interaction ($P < 0.585$) for ACa concentrations. Pooled, mean concentrations were increased ($P < 0.001$) by 4-6% throughout exercise and, despite reducing slightly at R0.5, remained significantly increased ($P < 0.001$) from BASE (Figure 6.11, Panel B). ACa concentrations remained increased ($P < 0.001$) from R1.0 to R3.0 with concentrations at R3.0 of 2.46 ± 0.06 $mmol \cdot L^{-1}$ in HIGH, 2.41 ± 0.06 $mmol \cdot L^{-1}$ in MOD and 2.40 ± 0.07 $mmol \cdot L^{-1}$ in LOW. Concentrations were not different from BASE at FU1 or thereafter.

PO_4: There was a significant main effect of *Time* ($P < 0.001$) but no significant *Condition* x *Time* interaction ($P = 0.236$) for PO_4 concentrations. Pooled, mean concentrations were significantly increased ($P < 0.001$) at EX20 by 13-21% from BASE and remained increased ($P < 0.001$) at EX60 (Figure 6.11, Panel C). Concentrations declined rapidly in recovery and were significantly lower ($P < 0.001$) than BASE at R1.0 to R3.0. The lowest PO_4 concentrations occurred at R2.0 in all groups. At this point concentrations were reduced by 7% in LOW and MOD and 17% in HIGH compared with BASE. Concentrations were not different from BASE at FU1 or thereafter. When measured from BASE to EX20, there was no significant correlation between the percentage changes in PO_4 and PTH in the LOW (Figure 6.12, Panel A), MOD (Figure 6.12, Panel B) and HIGH (Figure 6.12, Panel C) conditions. There were also no significant correlations between changes in PO_4 and PTH at EX40 or EX60 in any condition (data shown in Appendix B).

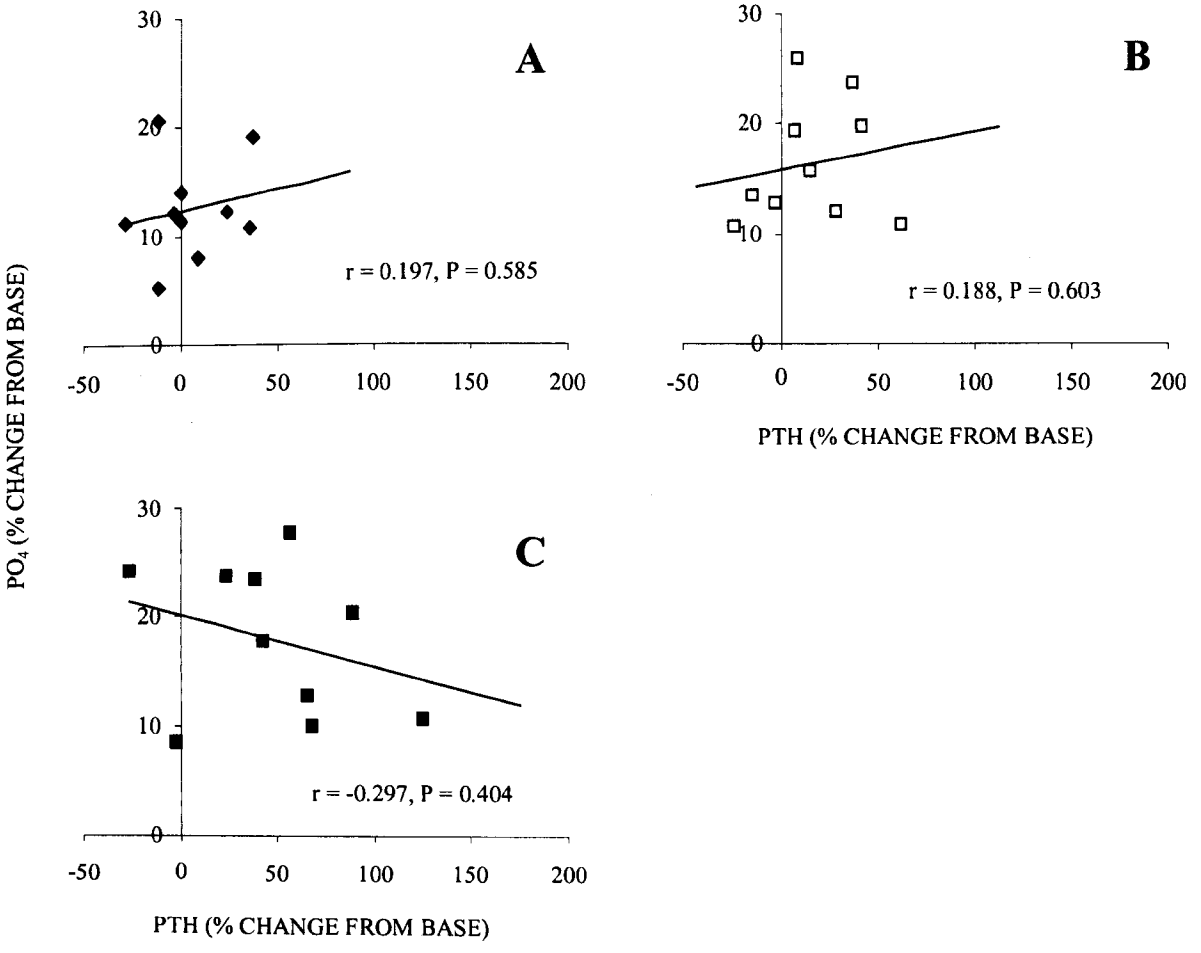

Figure 6.12. Relationship between the percentage changes in PO_4 and PTH concentrations measured from BASE to EX20 in the LOW (Panel A), MOD (Panel B) and HIGH (Panel C) conditions. * significant ($P < 0.05$).

6.4 Discussion

The main findings from this study are that 1) β-CTX concentrations were significantly higher with exercise at 75% VO_{2max} compared with 65% and 55% VO_{2max} in the first hour post exercise only; 2) there was no effect of exercise intensity on bone formation markers although bone ALP was increased in all groups on the third and fourth follow-up days; 3) when corrected for urinary Cr, both fPYD and fDPD tended to be higher on the third follow-up day although, at this time, there was also a tendency for mean Cr concentrations to be reduced; 4) fPYD and fDPD output were not significantly affected by any of the exercise protocols; 5) PTH was unchanged with exercise at 55% and 65% VO_{2max} but was transiently increased during exercise at 75% VO_{2max} and; 6) both ACa and PO_4 were transiently increased during exercise but there was no effect of cardiovascular exercise intensity.

There was no significant increase in β-CTX concentrations in any condition and concentrations decreased in the 3 h post-exercise period when compared with baseline. Zittermann *et al.* (2002) also observed a reduction in β-CTX at 3 h following a 60 min run at 70% of lactate threshold while a similar decrease (45%) was also shown in a resting control group. The magnitude of these decreases, and the time period over which they took place (0830 h to 1330 h), are comparable to that seen in the present study (39-42% decrease from 0800 h to 1215 h across all conditions). These results are consistent with the circadian rhythm of β-CTX in the late morning and early afternoon (Schlemmer and Hassager, 1999; Wichers *et al.*, 1999). Taken together, these findings suggest that 60 min of running up to 75% VO_{2max} has no effect on β-CTX, or possibly that any increase is, to some degree, masked by the variation in the β-CTX circadian rhythm.

In HIGH, following a small initial decline, β-CTX concentrations began to increase after 20 min of exercise and the highest concentrations occurred in the first hour post-exercise. This pattern of increase is similar to that reported by Guillemant *et al.* (2004) with 60 min of cycling at 80% VO_{2max}, where β-CTX began to increase after 30 min of exercise and peaked at 1-2 h post-exercise. In contrast to Guillemant *et al.* (2004), however, who reported a 45-50% increase in β-CTX, the increase from EX20 to 1 h post-exercise in the present study was only 7%, and from BASE, only 3%. One explanation for this discrepancy might be the difference in the acute nutritional status of the subjects in the two studies. In contrast to the present study, where subjects were fasted prior to and throughout exercise and recovery, Guillemant *et al.* (2004) fed subjects 3 h prior to exercise. Thus, prior to exercise, the suppressive effect of feeding on β-CTX would likely have been maximally expressed (Henriksen *et al.*, 2003) with β-CTX concentrations at, or close to, a nadir (Christgau, 2000; Bjarnason *et al.*, 2002). This may have produced a greater increase in β-CTX with exercise as the increase would no longer be 'masked' by the circadian rhythm that would result in continually declining concentrations in the late morning.

β-CTX concentrations in HIGH were 18% and 13% higher than in LOW and MOD in the first hour post exercise. The magnitude of this difference compares favourably with the difference in concentrations reported by Maïmoun *et al.* (2006) who observed a 20% difference in β-CTX immediately after exercise between exercise at 115% and 85% of VT, although in their study this difference was no longer evident at 15 min post-exercise. Herrmann *et al.* (2007) failed to standardise the time of day of exercise and the nutritional status of their subjects, but report increased β-CTX at 3 h and 24 h after exercise at 110%, but not 75%, of AT. In the present study, β-CTX concentrations were significantly lower than BASE at R3.0 and not significantly different from baseline on the four follow-up days. Therefore, any impact of cardiovascular exercise intensity on β-CTX concentrations was both modest and transient.

Neither Herrmann *et al.* (2007) nor Maïmoun *et al.* (2006) report their cardiovascular exercise intensities relative to VO_{2max} so direct comparisons with the present study cannot be made. Maïmoun *et al.*, (2006) do, however, report mean work load at VT was approximately 55% of W_{max}. As W_{max} and VO_{2max} during cycling are equivalent (Arts and Kuipers, 1994), it can be estimated that the 115% VT condition would have corresponded to approximately 60-65% VO_{2max}. As the largest difference between β-CTX concentrations occurred between 75%, and 65% and 55% VO_{2max}, this might indicate that the cardiovascular exercise intensity above which exercise stimulates β-CTX concentrations is higher in running than in cycling although the modest effect in the present study does not allow any firm conclusion to be drawn.

A threshold for the stimulation of β-CTX between 65% and 75% VO_{2max} might explain the different responses in Studies I and II. However, although the more prolonged, exhaustive nature of the exercise protocol in Study I might have accentuated any influence of cardiovascular exercise intensity, based on the results of the present study, cardiovascular exercise intensity alone seems unlikely to explain the different β-CTX responses seen in Studies I and II.

The present study is the first to make frequent measures of P1NP during, and in recovery from, acute exercise. The transient increase in P1NP during running is in contrast to 30 min of walking in healthy pre-menopausal women (Tosun *et al.*, 2006) or 30 min cycling at 95% VT in adolescent boys (Pomerants *et al.*, 2008) neither of which increase P1NP immediately after exercise, while Herrmann *et al.* (2007) report reduced P1NP concentrations at 3 h after low (75% of AT) cardiovascular intensity exercise. Despite the limitations of the study of Herrmann *et al.* (2007), their results do suggest a role for cardiovascular exercise intensity in the P1NP response to exercise, with higher intensity exercise resulting in small increases in P1NP at 3 and 24 h post-exercise. Although there was no effect of cardiovascular exercise intensity on the increase in P1NP in the present study, there was a tendency for a larger increase in P1NP in HIGH (30%) compared with in LOW and MOD (10 to 15%) at the end of exercise. Given the progressive rise in P1NP during exercise in HIGH and its rapid return to baseline with the termination of exercise, exercise duration might be a determining factor in the

magnitude of the response and, with longer duration exercise, a significant effect of cardiovascular exercise intensity might become apparent.

As the increase in P1NP was rapid, transient and occurred despite little evidence of an increase in β-CTX, it is unclear if the increase represents a direct effect of exercise on bone formation. As P1NP is cleared via scavenger liver receptors (Melkko *et al.*, 1994), the increase is unlikely to be explained by the accumulation of P1NP in the circulation due to changes in kidney function during exercise. P1NP is also unregulated in both skeletal muscle (Crameri *et al.*, 2004) and tendon tissue (Miller *et al.*, 2007; Hansen *et al.*, 2008) with exercise, so a contribution from these tissues to the increase in P1NP cannot be excluded. If so, the tendency for the increase in P1NP to be greater with increasing cardiovascular exercise intensity might reflect the accelerated efflux of type 1 collagen from tissues including the muscle, tendon and bone, resulting from a cardiovascular exercise intensity-dependent increase in blood flow.

In contrast to P1NP, changes in OC were modest and concentrations were reduced in the early part of exercise and again in the first few hours of the recovery period. Unlike other BTM, the circadian variation in OC is associated with the circadian variation in cortisol, with a nocturnal rise in cortisol preceding the daytime fall in OC concentrations (Nielsen *et al.*, 1992; Heshmati *et al.*, 1998). The circadian variation might therefore explain the significant reduction in OC concentrations seen in the early part of exercise, and also the reduced concentrations at 2 h and 3 h post-exercise. If so, the return of OC to baseline values by EX60 might point to a genuine effect of exercise. Increases in OC are reported in some (Thorsen *et al.*, 1997) but not all (Rudberg *et al.*, 2000; Tosun *et al.*, 2006) studies of low to moderate cardiovascular intensity endurance exercise, while no change is seen with short-term high intensity exercise (Rong *et al.*, 1997; Rudberg *et al.*, 2000; Ehrnborg *et al.*, 2003; Maïmoun *et al.*, 2005). In contrast, moderate duration (50-60 min), high intensity, endurance exercise increases OC (Maïmoun *et al.*, 2006; Herrmann *et al.*, 2007), while 30 min of incremental exercise does not (Wallace *et al.*, 2000), suggesting that both exercise duration and cardiovascular intensity might be determinates of the OC response to exercise.

Data from Herrmann *et al.* (2007) and Maïmoun *et al.* (2006) both suggest an effect of cardiovascular exercise intensity on changes in OC, with concentrations only increased with high intensity exercise. There was no effect of cardiovascular exercise intensity on OC in the present study although there was a tendency for higher concentrations at the end of exercise in MOD and HIGH. However, while the present study tested for statistical differences between the three different intensities, the other studies only looked at changes across time within each intensity which may explain the differences between findings.

Given the observed relationship between cortisol and OC at rest, with increased cortisol preceding decreased OC, changes in OC with exercise might also be mediated by changes in cortisol. As observed elsewhere (Hill *et al.*, 2008), in the present study there was a threshold for the stimulation of cortisol concentrations but despite higher cortisol concentrations in HIGH compared with MOD and LOW there was no difference in OC responses. Herrmann *et al.* (2007) did not measure cortisol, but their results also point to higher OC levels at higher cardiovascular exercise intensities, while Maïmoun *et al.* (2006) showed an increase in OC at 115%, but not 85%, of VT, despite no change in cortisol in either condition. Taken together, these findings suggest that factors other than, or in addition to, cortisol influence OC during and immediately following acute exercise.

The increase in pooled, mean bone ALP at 3 and 4 days post-exercise suggests an increase in bone mineralisation with a single bout of running that is independent of increased cardiovascular exercise intensity. On the days that follow exercise, previous studies report unchanged bone ALP concentrations after brisk walking (Welsh *et al.*, 1997), a 5-30 km run (Brahm *et al.*, 1996) and a marathon (male runners only) (Malm *et al.*, 1993) and decreased concentrations after a marathon (female runners only) (Malm *et al.*, 1993) and a 246 km run (Mouzopoulos *et al.*, 2007). Following a single bout of resistance exercise bone ALP concentrations are either unchanged (Whipple *et al.*, 2004) or decreased (Ashizawa *et al.*, 1998). Thus, when measured in fasted, early morning samples, this study is the first to observe a sustained increase in bone ALP after a single bout of exercise.

There was no effect of cardiovascular exercise intensity on fPYD/Cr and fDPD/Cr, although there was an overall effect of exercise in increasing their concentrations due to the tendency for increased concentrations on the third follow up day. In contrast, Brown *et al.* (1997) report no effect of a single bout of eccentric exercise on fPYD in FMV samples up to 9 days post-exercise and Welsh *et al.* (1997) report a marked increase in fPYD and fDPD in 24 h samples collected on the day of, and on the day after, a 30 min walk.

In contrast to Studies I and II, mean Cr concentrations were not significantly increased by exercise with both increased and decreased concentrations in individual subjects at FU1. With increasing cardiovascular exercise intensity, a greater number of subjects showed increases in Cr but subsequently, there continued to be considerable variability in individual Cr concentrations and even a tendency for a decrease in mean concentrations at FU3. There are no previous reports of a decrease in mean concentrations. However, as the decrease in Cr was of a similar magnitude (5 to 10%) to the increase in mean fPYD/Cr and fDPD/Cr concentrations at FU3, the increase in Cr-corrected pyridinolines might be an artefact of the decrease in Cr.

While Cr-corrected pyridinolines tended to be increased by exercise, fPYD and fDPD outputs were not significantly different from BASE at FU1 through to FU4. As β-CTX concentrations were also unchanged, and there was no significant effect of exercise on mean urine volume, measuring

pyridinoline output might be considered to be superior to measuring Cr-corrected pyridinolines. However, the lack of change in mean urine volume masks considerable variations in volume both between and within individual subjects and above and below BASE values, which is reflected in the large variability in the pyridinoline output at each time point. Thus, the constant mean urine volume might mistakenly be attributed to a lack of day-to-day variation in urine volume in a SMV. As with Cr, such variability might mask a genuine change in fPYD and fDPD and the usefulness of pyridinoline output in a SMV as a measure of changes in bone resorption under these conditions is, therefore, unclear.

The current study is the first to examine the effect of cardiovascular exercise intensity on changes in OPG with acute running and finds no effect of increasing intensity from 55% to 75% VO_{2max}. Despite increasing basal OPG concentrations in the late morning (Joseph *et al.*, 2007), as in Study I, the magnitude of this increase (15-41%) and the short time scale (BASE to EX20 = 35 min) over which it takes place, suggests that much of the increase in OPG is a genuine response to exercise. Additionally, the 25% increase in the MOD condition compares well with the magnitude (20-27%) of the increase in Study I after FD. Although the increase in OPG tended to be more rapid and marked with increasing cardiovascular exercise intensity, this finding also indicates that the increase in OPG occurs during 60 min of running at only 55% VO_{2max}. This finding suggests that cardiovascular exercise intensity alone does not explain the lack of any change in OPG reported during a 15.8 km run lasting 1.26 ± 0.19 h (Ziegler *et al.*, 2005), as the average exercise intensity in the study is likely to have been greater than 55% VO_{2max}.

Although little is known about the relationship between changes in β-CTX and OPG along short time scales similar to that in the present study, in circadian rhythm studies the strongest relationship between OPG and β-CTX exists when changes in OPG precede changes in β-CTX by 2-3 h (Joseph *et al.*, 2007). Joseph *et al.* (2007) also conclude that the dynamic change in OPG concentration, rather than the absolute concentration, may be the important regulatory factor for osteoclast activity. Thus, the tendency for β-CTX concentrations to be higher in HIGH than LOW and MOD in the first hour post-exercise might be related to the more dynamic change in OPG in HIGH in the early part of exercise. Although RANKL was not measured, the rapid increase in OPG might be responding to a more dynamic change in RANKL and bone resorption, which is subsequently reflected in β-CTX concentrations. The small differences between changes in OPG and β-CTX concentrations in the three conditions, however, do not allow any firm conclusion to be drawn.

Increased PTH concentrations in HIGH, but not MOD or LOW, suggests that the cardiovascular intensity of exercise is a determinant of the increase in PTH during a bout of acute running. Previously, Salvesen *et al.* (1994) observed no increase in PTH until the 4th stage of a five stage, 40 min incremental treadmill run, with concentrations increased by 50% during the 4th stage and by 60% by the end of the test. During 50 min of steady-state cycling, Maïmoun *et al.* (2006) showed that

PTH concentrations were unchanged with exercise at 85% of VT but increased by 41% at 115% of VT. Together these findings suggest the presence of a threshold below which PTH concentrations are not increased during acute endurance exercise.

Neither of these previous studies report their cardiovascular exercise intensities relative to VO_{2max} so direct comparisons with the current study regarding the possible location of this threshold are not possible. However, mean heart rate at the end of stage four in the study of Salvesen *et al.* (1994) was 174 ± 11 bpm which is comparable with that in the present study in the HIGH condition (177 ± 11), as was the age range of their subjects (27-35 y). In contrast, as discussed above, cardiovascular exercise intensity at 115% VT in the study of Maïmoun *et al.* (2006) would be equivalent to only 60-65% VO_{2max}. Together, these findings might suggest that the threshold for the effect of cardiovascular exercise intensity on PTH concentrations is lower in cycling than in running.

A lower threshold in cycling might explain why PTH is increased after cycling at 60-75% VT (Barry and Kohrt, 2007). As subjects in the study of Barry and Kohrt, (2007) cycled for 2 h, and PTH was only increased at the end of exercise in the study of Maïmoun *et al.* (2006), the duration of cycling exercise might be an important factor in the PTH response. In contrast, in the present study, PTH concentrations increased after only 20 min of running at 75% VO_{2max}, while Guillemant *et al.* (2004) report a marked increase in PTH after 30 min of cycling at 80% VO_{2max}. Thus, it is possible that a higher cardiovascular intensity exercise also promotes a more rapid increase in PTH concentrations.

Although the prompt increase in PTH in the present study is similar to that observed in Study I, in Study I, and indeed Study II, mean PTH concentrations were increased with exercise at 65% VO_{2max}, whereas the MOD condition had no effect on PTH in the present study. The reason for this discrepancy is not clear as, compared to Study I, exercise was of identical duration (60 min), was performed at the same time of day, and both sets of subjects were fasted prior to exercise. The physical characteristics of subjects were also similar and, although subjects ranged in their level of aerobic fitness, the results of Study I suggest that training status is not a factor in the PTH response. One possibility is that there is a high degree of variability in the individual PTH response to running. In the present study, one subject showed only a 12% increase in PTH in HIGH, whereas two subjects showed an increase in LOW of more than 30%, while PTH increased 50% or more in three subjects in MOD. Interestingly, as cardiovascular exercise intensity increases, the direction of change in PTH appears to become more consistent, with six subjects showing decreased PTH concentrations in LOW, two in MOD but none in HIGH. Thus, it is possible that factors in addition to cardiovascular exercise intensity might also be mediating the PTH response to acute exercise.

As in Study I and Study II, PTH concentrations were decreased from pre-exercise levels in the early post-exercise period. Again, the magnitude and rate of the decline is much greater than would be expected than from the circadian rhythm alone (Fuleihan *et al.*, 1997) suggesting a genuine effect of exercise. This idea is supported by different responses seen in the three experimental conditions, with PTH levels unchanged in LOW, decreased 20% in MOD and 35% in HIGH. As in Study I this might be related to the increase in ACa concentrations which tended to be larger in HIGH (5-6%) compared with MOD and LOW (2-4%).

Unlike Studies I and II, where there were transient increases in PTH but no change in bone formation markers, in the present study there was a transient increase in PTH in HIGH and an increase in bone ALP concentrations 3 and 4 days post-exercise. Although this might suggest an anabolic effect of PTH during exercise (Qin *et al.*, 2004) there was no effect of cardiovascular exercise intensity on bone ALP and, if anything, the increase tended to be largest in the MOD condition where there was no increase in mean PTH concentrations. In addition, of the three subjects who displayed marked increases in PTH during exercise in MOD, two of them had no marked change in bone ALP concentrations at FU3 and FU4. Therefore, at least up to 4 days post-exercise, there is no evidence that the transient increase in PTH with acute running is associated with an anabolic effect on bone.

Although the difference was modest, modest, β-CTX concentrations in HIGH were higher than in MOD and LOW in the first hour post-exercise, which might suggest that, rather than bone formation, the increase in PTH might have had a modest stimulatory effect on bone resorption. In circadian rhythm analysis, the strongest relationship between changes in PTH and changes in β-CTX is observed when β-CTX lags PTH by 2 h (Joseph *et al.*, 2007). In the present study, PTH was increased after 20 min of exercise and the maximum difference in β-CTX concentration was evident by the end of exercise, 40 min later. Although this is a much shorter time period that that reported by Joseph *et al.* (2007), this might be explained by the more dynamic change in PTH with exercise. PTH levels rapidly returned to baseline following exercise and, by 2 h post-exercise, β-CTX concentrations were similar in all three conditions. This might be further evidence of a direct effect of changes in PTH on osteoclast activity.

The increase in PTH concentrations in only the HIGH condition is not explained by a decrease in serum calcium concentrations, as ACa was increased during exercise. Similarly, Maïmoun *et al.* (2006) reported increased PTH with exercise at 115% but not 85% VT despite no change in total calcium at either intensity. Interestingly, ACa was also increased in the LOW and MOD conditions but there was no change in PTH. This further suggests that serum calcium concentrations do not mediate changes in PTH during exercise and also that the increase in ACa during exercise is not a result of mobilisation from bone as a result of increased PTH-induced bone resorption. Ionised calcium was not measured in the present study but the available evidence points to increasing, rather than decreasing, iCa with increasing intensity (Ljunghall *et al.*, 1984b; Rong *et al.*, 1997).

Such an effect would be consistent with an increase in blood lactate and the onset of acidosis decreasing the binding of calcium to albumin (López *et al.*, 2004).

Blood pH was not measured in the present study so the presence of metabolic acidosis, particularly in the HIGH condition, cannot be excluded. However, blood pH remains unchanged during endurance exercise that results in higher lactate concentrations than those seen in this study (Peinado *et al.*, 2006), while PTH is reported to increase despite no change in pH (Rudberg *et al.*, 2000; Guillemant *et al.*, 2004). As with PTH, the accumulation in catecholamines in the circulation is related to cardiovascular exercise intensity with a significant increase occurring at exercise intensities similar to that of the lactate and ventilatory thresholds (Mazzeo and Marshall, 1989; Weltman *et al.*, 1994). Although physiologically-relevant increases in catecholamines have previously been reported to have no effect on PTH (Body *et al.*, 1983), this relationship requires a re-examination with more sensitive PTH assays and an increase in catecholamines as the factor mediating the increase in PTH in the HIGH condition cannot be excluded.

Like the majority of previous studies of acute, endurance exercise (Ljunghall *et al.*, 1986; Nishiyama *et al.*, 1988; Tsai *et al.*, 1997; Rudberg *et al.*, 2000; Guillemant *et al.*, 2004; Maïmoun *et al.*, 2006), Studies I and II showed that an increase in PTH is accompanied by an increase in PO_4. In the present study however, PTH increased significantly only in the HIGH condition, despite similar increases in PO_4 in all three conditions. This corroborates the findings of Maïmoun *et al.* (2006) who report increased PTH with exercise at 115%, but not 85%, of VT despite similar increases in PO_4. Together, these findings suggest that the increase in PTH is not related to the increase in PO_4. In both the present study, and that of Maïmoun *et al.* (2006), the increase in PO_4 did tend to be greater with increasing cardiovascular exercise intensity suggesting the possibility of a threshold effect for PO_4 on PTH. However, in the present study, there was no significant correlation between the increases in PO_4 and PTH at any time point during exercise in the HIGH condition. In addition, the individuals who showed marked increases in PTH with exercise in the LOW and MOD conditions did not display the largest increases in PO_4. Taken together, these results suggest that in the present study at least, the increase in PTH with acute running at 75% VO_{2max} is unlikely to be mediated by increased PO_4.

Finally, it has been suggested that increases in PO_4 with exercise might increase PTH indirectly by reducing iCa levels as a result of changes in complex-binding (Nishiyama *et al.*, 1988). Thus, by showing different PTH responses, despite similar increases in PO_4, the results of the present study provide further indirect evidence that a decrease in iCa is unlikely to explain the increase in PTH concentrations at 75% VO_{2max}.

An important consideration for this particular study is the possible role of changing mechanical loads in the findings, particularly for bone turnover markers. With this design, it is not possible to distinguish between the effects of increasing cardiovascular exercise intensity itself and the associated increase in mechanical loads on bone – likely from a combination of muscular forces and GRFs – that occur with increasing running speed (Burr *et al.*, 1996). This is of particular importance because the manner in which bone responds to loading is dependent on both the magnitude of strain, with bone formation only occurring above a threshold level of strain (Chow *et al.*, 1993; Rubin *et al.*, 1985; Turner *et al.*, 1994), but excessive strains resulting in damage and bone resorption (Bentolila *et al.*, 1998; Mori and Burr, 1993). Using accelerations and the slope of the acceleration signal as surrogates for strain and strain rate, population-based exercise intervention studies in humans report similar findings, with physical activitites inducing acceleration levels exceeding approximately 4*g* (Vainionpää *et al.*, 2006; Jämsä *et al.*, 2006) and slopes greater that 1000 $m \cdot s^{-3}$ (Heikkinen *et al.*, 2007) correlating with changes in hip BMD. In these studies, the threshold for acceleration level was generally exceeded with running at 13 $km \cdot h^{-1}$ (mean = 4.25 g, range = 3 – 5.25 g), but not at 9 $km \cdot h^{-1}$ (mean = 3 *g*, range = 2 – 4.5 *g*) (Vainionpää *et al.*, 2006), whereas the threshold for acceleration slope was generally exceed at both 9 $km \cdot h^{-1}$ (mean = 1200 $m \cdot s^{-3}$, range = 700 – 1700 $m \cdot s^{-3}$) and 13 $km \cdot h^{-1}$ (mean = 1400 $m \cdot s^{-3}$, range = 800 – 1800 $m \cdot s^{-3}$) (Heikkinen *et al.*, 2007). In contrast, no data exists as to the magnitude of strain or strain rates that induce bone damage and remodelling in humans.

Data reported in the studies of Jämsä *et al.*, (2006) and Vainionpää *et al.*, (2006) was collected from female subjects (aged 20–58 years, BMI 19.1 – 29.7 $kg \cdot m^2$) and using a waist-mounted accelerometer making comparisons with previous literature difficult, as these studies have typically used male subjects and tibial-mounted accelerometers (Verbitsky *et al.*, 1998; Mizrahi *et al.*, 2000; Derrick *et al.*, 2002; Mercer *et al.*, 2002: Mercer *et al.*, 2003). For example, Verbitsky *et al.*, (1998) report mean acceleration values ranging from 6-9 *g* at 9.9 $km \cdot h^{-1}$. Mizrahi *et al.*, (2000) report mean accelerations in excess of 6 *g* during 12.7 ± 0.7 $km \cdot h^{-1}$ treadmill running with Derrick *et al.*, (2002) reporting similar values (6.11 – 7.38 *g*) at 12.2 ± 0.4 $km \cdot h^{-1}$. Mercer *et al.*, (2002) measured mean values of 6.1 *g*, 6.1 *g* and 7.2 *g* with treadmill running at 11.5 ± 1.1 $km \cdot h^{-1}$, 13.7 ± 1.1 $km \cdot h^{-1}$ and 16.2 ± 1.4 $km \cdot h^{-1}$. In a subsequent study, however, they measured only 5.0 – 5.3 *g* at 13.7 $km \cdot h^{-1}$ using a group of subjects with a similar mean body mass (Mercer *et al.*, 2003).

It is likely that accelerations at the lower limb in the studies of Jämsä *et al.*, (2006) and Vainionpää *et al.*, (2006) would be greater than those measured at the waist, as the magnitude of accelerations during running is attenuated at skeletal sites more distant from the point of impact (*i.e.* the foot) (Mercer *et al.*, 2002, Mercer *et al.*, 2003). How much greater, however, is not clear. The only informative data in this respect comes from a single subject in a study by Voloshin *et al.*, (1998) who report accelerations at the sacrum to be only one third of those measured at the tibia during running at 9.9 $km \cdot h^{-1}$. However, the skin-mounted accelerometers used in these studies likely underestimate the true

magnitude of bone strain (Lafortune, 1991), while much lower accelerations (1.1-2.4 g) and acceleration slopes (< 1000 $m \cdot s^{-3}$) may stimulate bone formation in the calcaneus (Vainionpää *et al.*, 2006; Heikkinen *et al.*, 2007).

It is likely, therefore, that the accelerations and acceleration slopes required to induce osteogenesis were achieved in the current study. However, it is also possible that they occurred more frequently and at more skeletal sites in the HIGH condition (treadmill velocity range = 9.7 – 13.4 $km \cdot h^{-1}$) compared with the MOD (range = 9.3 – 11.7 $km \cdot h^{-1}$) and LOW (range = 7.0 – 10.1 $km \cdot h^{-1}$) conditions. As a result, the osteogenic stimulus of running due to mechanical loading alone may have been different between the three conditions, which might, in part, have contributed to the findings.

In conclusion, increasing cardiovascular exercise intensity from 55% to 75% VO_{2max} during 60 min of running in fasted men was associated with higher β-CTX concentrations in the first hour post exercise, although the effect was modest. Cr-corrected pyridinolines tended to increase on the recovery days although the largest increase coincided with a significant reduction in mean Cr concentrations. When pyridinoline output was calculated, fPYD and fDPD were not different from baseline on the four recovery days although this masked considerable individual variations in urine volume and thus calculated pyridinoline output. Bone ALP concentrations increased at 3 and 4 days post-exercise suggesting a beneficial effect of a single bout of this type of exercise on bone mineralisation. Exercise resulted in a rapid increase in OPG concentrations which was not influenced by cardiovascular exercise intensity, while PTH concentrations were transiently increased only at 75% VO_{2max}, suggesting a threshold below which exercise has no effect. The increase in PTH only with exercise at 75% VO_{2max} cannot be explained by changes in serum calcium and is unlikely to be mediated by increased PO_4 concentrations.

CHAPTER VII

STUDY IV – THE EFFECT OF PRE-EXERCISE FEEDING ON THE METABOLIC RESPONSE OF BONE TO ACUTE RUNNING

7.1 Introduction

The results of Study III suggest that the effect of exercise on bone resorption is, in part, dependent on cardiovascular exercise intensity. The tendency for higher post-exercise β-CTX concentrations in the HIGH condition compared with the MOD and LOW conditions suggests a threshold for this effect might exist between exercise intensities of 65% and 75% VO_{2max}. This effect might, in part, explain the different β-CTX responses in Study I and Study II, as in Study I cardiovascular exercise intensity exceeded 65% VO_{2max} during intermittent exhaustive running. However, this difference was modest and transient, and the lack of any effect of increasing cardiovascular exercise intensity on β-CTX when measured on the four follow-up days, suggests that exercise intensity alone fails to fully explain the increased β-CTX concentrations in Study I but not Study II.

Another potentially important difference between Studies I and II is the nutritional status of subjects prior to the onset of exercise. In Study I, exercise was conducted after an overnight fast and all food intake (except plain water) was prohibited until 2 h after exercise was completed. Study II, however, was designed to represent an ecological valid, two day schedule including exercise sessions and meals. As such, subjects consumed a standardised breakfast at 0815 h and further meals at 1145 h, 1545 h and 1930 h. As a result, all exercise bouts were completed after the ingestion of one or more meals.

The ingestion of food as individual macronutrients (Bjarnason *et al.*, 2002; Henriksen *et al.*, 2003; Holst *et al.*, 2007) or as a mixed meal (Bjarnason *et al.*, 2002; Clowes *et al.*, 2002a) has been shown to suppress resting β-CTX concentrations. Time-course studies indicate that the effect of feeding occurs within 1 h and, when maximally expressed, reduces β-CTX concentrations by around 50% (Bjarnason *et al.*, 2002; Henriksen *et al.*, 2003; Holst *et al.*, 2007). In contrast to bone resorption markers, markers of bone formation are either unaffected (Bjarnason *et al.*, 2002; Henriksen *et al.*, 2003) or show a small reduction (Clowes *et al.*, 2002a) following nutrient ingestion, resulting in a positive, albeit transient, bone remodelling balance. Like food, acute calcium ingestion also results in a rapid and marked suppression of resting β-CTX concentrations (Guillemant *et al.*, 2000; Zikán *et al.*, 2001) and, when ingested before and during strenuous exercise, abolishes the increase in β-CTX observed during and immediately after a subsequent bout of acute, endurance exercise (Guillemant *et al.*, 2004).

The available evidence suggests that food intake prior to exercise might, in a similar manner to calcium, attenuate the increase in bone resorption associated with strenuous exercise. If so, this effect might, in part, explain the different β-CTX responses to Studies I and II. The attenuation of the

exercise-associated increase in bone resorption by feeding might also represent a useful intervention during periods of training to minimise any negative effects on bone.

The aim of this study was to investigate the effect of feeding with a single mixed meal on the bone metabolic response to a subsequent bout of exercise. It was hypothesised that the suppressive effect of food ingestion on β-CTX concentrations would attenuate the increase in bone resorption associated with subsequent, acute exercise.

7.2 Materials and Methods

7.2.1 Subjects

Ten men were recruited to participate in the study (Table 7.1). All subjects were in good physical condition, with a history of weight-bearing exercise and average to above average levels of fitness. The group was a mixture of team sports players and recreational runners and included one 'club' level runner and one recreational mountaineer. All subjects performed at least bout of endurance running per week. The study had a within-subject design with all subjects completing all experimental conditions.

7.2.2 Overview of exercise intervention

On Day 4, all subjects performed one, 60 min bout of treadmill running at 65% following either an overnight fast (FAST) or a standardised breakfast (FED).

7.2.3 Pre-trial measurements

Medical Screening: Subjects underwent a full medical examination as described in Section 3.2.1.

Dietary Analysis: Subjects completed a three-day food diary as described in Section 3.2.2.

Determination of the association between oxygen uptake and running velocity during level (0^o gradient) treadmill running and of maximal rate of oxygen uptake: The association between oxygen uptake and running velocity during level running was determined as described in Section 3.2.3. Maximal rate of oxygen uptake was determined as described in Section 3.2.4. The results of the two tests were used to estimate treadmill velocities required to elicit 50% and 65% VO_{2max} estimated based on the regression line of % VO_{2max} and treadmill velocity.

Experimental Dietary Provision: A diet consisting of 55% carbohydrate, 30% fat and 15% protein, and isocaloric with their habitual diet was designed for each subject based on individual dietary habits. This diet was isocaloric with their habitual diet (Table 7.3). Subjects were provided with three menus that were given in a three-day cyclic order with menu A on Days 1 and 5, Menu B on Days 2 and 6, and Menu C on Days 3 and 7 (for details of diet on Day 4 see *Section 7.2.4*). Details of menus for individual subjects can be found in Appendix A.

7.2.4 Trial procedures

Day 4: Following an overnight fast, subjects arrived at the laboratory at 0730, provided a second void urine sample and had their body mass measured (Mettler-Toledo ID7, Mettler-Toledo, Germany). Subsequently, subjects adopted a semi-recumbent position and a cannula (18GA 1.2x45 mm, Becton Dickinson, USA) was inserted into a vein in the forearm, where it remained until after the final blood sample. A baseline blood sample was collected at 0800 h and exercise commenced at 1030 h

(Figure 7.1). In the FED condition subjects received a standardised breakfast (2.3 MJ, 60% CHO, 32% FAT, 8% PRO, 116 mg Calcium) at 0815 h while in FAST they remained fasted. Subjects were asked to consume the meal in 5 to 10 min and mean consumption time was 7.6 (range 6.0 to 10.5) min.

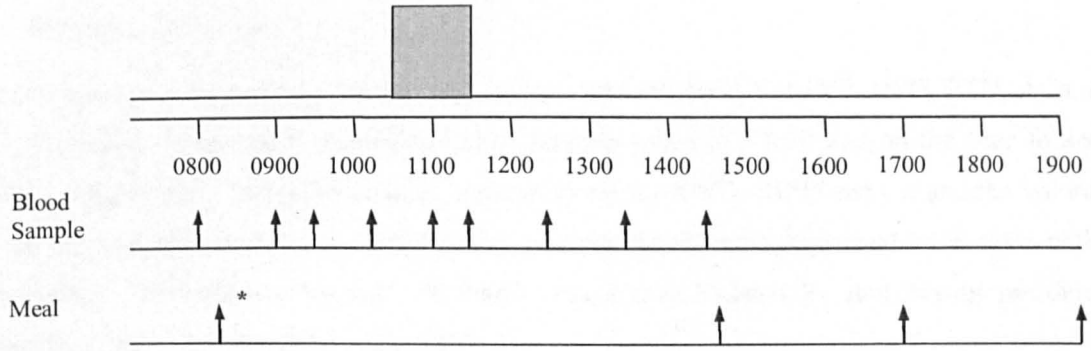

Figure 7.1. The experimental protocol on Day 4 in the FAST and FED conditions. Grey box indicates 60 min of treadmill running at 65% VO_{2max}. Arrows indicate blood samples and meal times; * Standardised breakfast provided in the FED condition only.

Exercise bouts consisted of 60 min of treadmill running at 65% VO_{2max} preceded by a 5 min warm-up at 50% VO_{2max}, separated by 5 min for volitional stretching. Sixty second samples of expired air and ratings of perceived exertion (RPE) were collected after 18, 38 and 58 min of exercise, and heart rate was recorded continuously. In the period between 0815 h and 1030 h, water was consumed *ad libitum* (0.12 ± 0.09 and 0.16 ± 0.09 L in FAST and FED) as it was during exercise (0.34 ± 0.16 and 0.31 ± 0.14 L in FAST and FED). The ambient room temperature during exercise was 19.7 ± 0.9 °C.

On completion of exercise, nude body mass was again measured, and subjects then rested in a semi-recumbent position in bed for a further 3 h. The difference between pre- and post-exercise body mass was calculated (0.80 ± 0.22 and 0.76 ± 0.25 kg in FAST and FED) and subjects consumed 1.5 ml of plain water for every gram change in body mass during the recovery period. Blood samples were collected at identical time points in the two conditions; at 0800 h (BASE), at 0900 h, 0930 h, 1015 h (Pre-exercise; PRE), after 30 (EX30) and 60 (EX60) min of exercise, and after 1 h (R1.0), 2 h (R2.0) and 3 h (R3.0) of recovery.

In each condition, subjects consumed a standardised diet (13.2 MJ, 53% CHO, 32% FAT, 15 % PRO, 767 mg calcium). In FED this was divided into four meals at 0815 h (the standardised breakfast), 1430 h, 1730 h and 2000 h and into three meals in FAST at 1430 h, 1730 h and 2000 h. The meal at 1430 h was eaten in the laboratory immediately following the final blood sample (R3.0) with the further two meals provided to subjects to be consumed at home (Figure 7.1). Only plain water was allowed after 2100 h.

Days 5 to 8 (FU1 – FU4): Subjects attended the laboratory at 0730 h, provided a second void urine sample and had their nude body mass was measured. At 0800 h a blood sample was collected. Only plain water was allowed after 2100 on the days prior to laboratory visits.

7.2.5 Biochemical analysis

All blood samples were analysed for glucose, lactate, cortisol, β-CTX, P1NP, OPG, PTH, ACa, PO_4, leptin and ghrelin. Bone ALP was measured in samples taken at BASE and on the four follow-up days (FU1 – FU4) only. All urine samples were analysed for fPYD, fDPD and Cr, and the volume of each sample was also measured. Pyridinoline concentrations were expressed as a ratio with Cr concentrations. Pyridinoline 'output' (in nmol) was also calculated by multiplying pyridinoline concentrations ($nmol \cdot L^{-1}$) by urine volume (L).

7.2.6 Statistical Analysis

All data are presented as mean ± 1SD unless otherwise stated. Statistical significance was accepted at an alpha level of $P < 0.05$. Paired samples t-tests were used to compare habitual and experimental dietary data and variables relating to exercise performance.

All biochemical data were analysed using a LMM, with the factors *Time* (of sampling) and *Condition* (FAST *vs* FED) included and with individuals as a random within-group factor. The assumptions of the LMM were investigated by examining the distribution of residuals and the pattern of residuals versus fitted values. Where non-normality or non-constant variance was observed, a transformation was applied to the data so that the assumptions were satisfied. ACa, PO_4 and cortisol did not require transformation. Normality and homogeneity were achieved following log transformations for all other variables.

Where there was a significant main effect of *Time* but no significant *Group* x *Time* interaction, each subsequent time point was compared against BASE using a pooled mean using Dunnett's test with BASE as the '*Control*'. When the *Group* x *Time* interaction was significant, within each group each subsequent time point was compared against BASE using Dunnett's test with BASE as the '*Control*' and groups were compared to each other at all time points using the SNK test.

Pearson's correlation coefficient was calculated between the percentage changes in PO_4 and PTH from BASE to EX30 in the FAST and FED groups.

7.3 Results

7.3.1 Subject characteristics

Subject characteristics are shown in Table 7.1

Table 7.1. Subject characteristics.

Measure	Value
n	10
Age (y)	28 ± 4
Body Mass (kg)	82.3 ± 6.4
Height (m)	1.78 ± 0.06
BMI ($kg \cdot m^{-2}$)	26.0 ± 3.1
VO_{2max} ($ml \cdot kg^{-1} \cdot min^{-1}$)	52.2 ± 6.3

Values are mean ± 1SD. BMI, body mass index.

7.3.2 Dietary analysis and experimental dietary provision

Dietary analysis: Energy intake, macronutrient composition and dietary calcium from food diary analysis are shown in Table 7.2.

Table 7.2. Habitual energy intake, macronutrient composition and dietary calcium content.

Variable	Value
Energy (MJ)	10.2 ± 2.8
CHO (g)	336.7 ± 96.6
CHO ($g \cdot kg\ FFM^{-1}$)	4.9 ± 1.7
CHO (% of total energy)	52.0 ± 8.6
Fat (% of total energy)	28.3 ± 5.9
Protein (% of total energy)	18.7 ± 3.8
Calcium ($mg \cdot day^{-1}$)	981 (range, 608 - 1816)

Values are mean ± 1SD unless otherwise stated. CHO, carbohydrate; FFM, fat-free mass.

Experimental dietary provision: The energy content and macronutrient composition of the experimental diets are shown in Table 7.3. There was no significant difference between habitual energy intake and that provided by the experimental diet ($P = 0.05$). Experimental diets provided a smaller quantity of protein (16.0 % *vs* 18.7%, $P < 0.05$) but there were no significant differences between any other habitual and experimental dietary variables.

Table 7.3. Energy content and macronutrient composition of the experimental diets consumed by subjects on Days 1-3 and 5-7 of each experimental condition.

Measure	Value
Energy (MJ)	10.5 ± 2.5
CHO (g)	362.5 ± 83.0
CHO (g·kg FFM^{-1})	5.5 ± 1.5
CHO (% of total energy)	54.7 ± 1.5
FAT (% of total energy)	29.3 ± 1.3
PRO (% of total energy)	16.0 ± 1.4 [a]
Calcium (mg·day^{-1})	954 (range, 755 - 1306)

Values are mean ± 1SD unless otherwise stated. CHO, carbohydrate; FFM, fat-free mass. [a] different ($P < 0.05$) from habitual diet.

7.3.3 Baseline biochemistry

There were no significant differences in baseline values of any biochemical measure between the FAST and FED groups. Baseline PYD/Cr and ghrelin concentrations tended to be higher in FED compared with FAST although these differences did not reach the assigned level of statistical significance ($P = 0.051$ and $P = 0.053$ respectively) (Table 7.4).

Table 7.4. Baseline biochemistry in the FAST and FED groups.

Marker	FAST	FED	P Value (paired samples t-test)
β-CTX (ug·L^{-1})	0.56 ± 0.24	0.52 ± 0.23	0.118
P1NP (ug·L^{-1})	53 ± 24	52 ± 22	0.672
OC (ug·L^{-1})	26.7 ± 8.7	26.6 ± 8.4	0.881
Bone ALP (U·L^{-1})	19 ± 7	21 ± 7	0.133
Cr (umol·L^{-1})	16.6 ± 10.1	15.0 ± 5.1	0.611
fPYD/Cr (nmol·mmol Cr^{-1})	16.1± 2.7	18.0 ± 3.5	0.051
fDPD/Cr (nmol·mmol Cr^{-1})	4.1± 0.6	4.4 ± 0.6	0.089
OPG (pmol·L^{-1})	1.7 ± 0.8	1.8 ± 0.8	0.096
PTH (pmol·L^{-1})	4.4 ± 1.6	4.7 ± 1.6	0.334
ACa (mmol·L^{-1})	2.27 ± 0.07	2.26 ± 0.10	0.788
PO_4 (mmol·L^{-1})	1.06 ± 0.09	1.07 ± 0.13	0.860
Leptin ng·mL^{-1})	5.3 ± 2.7	5.3 ± 3.0	0.872
Ghrelin ng·L^{-1})	110 ± 28	128 ± 45	0.053
Cortisol (nmol·L^{-1})	370 ± 89	348 ± 80	0.507

Values are mean ± 1SD.

7.3.4 Body Mass

There was no significant main effect of *Time* (P = 0.275) and no significant *Condition* x *Time* interaction (P = 0.974) for body mass measured from Day 4 (BASE) to Day 8 (FU4) (Figure 7.2).

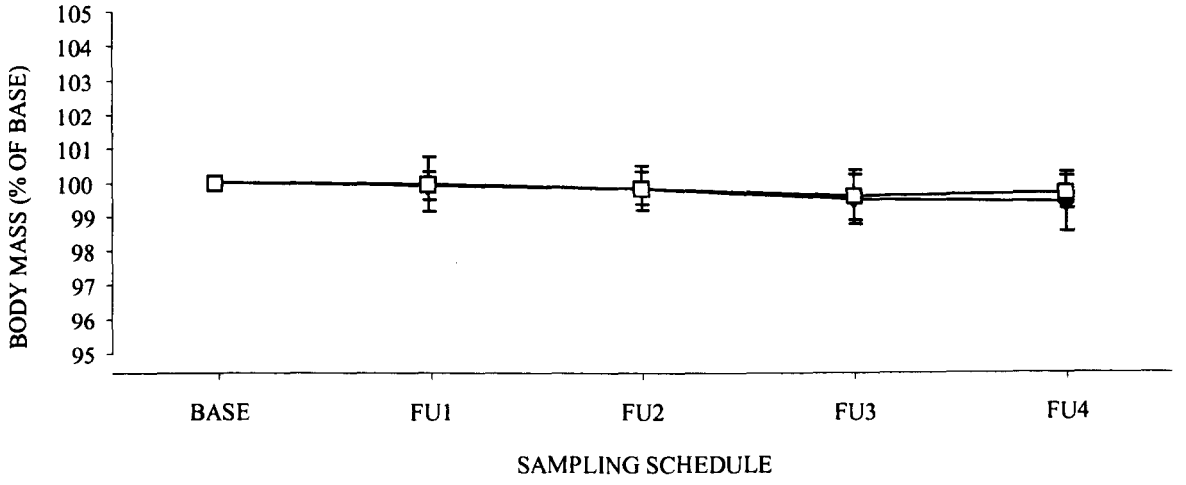

Figure 7.2. Percentage change in body mass from baseline (BASE) on the four follow-up days (FU1 – FU4) in the FAST (filled diamonds) and FED (open squares) conditions.

7.3.5 Exercise variables

Exercise intensities in the FAST and FED conditions were 64.9 ± 2.2% and 64.5 ± 3.3% VO_{2max}. There was no difference in VO_2 or RPE during exercise between the FAST and FED conditions. Heart rate, expressed as a percentage of maximum heart rate (HR_{max}), was higher (P < 0.001) in FAST compared with FED (84 ± 5% vs 79 ± 8%) as was respiratory exchange ratio (RER) (0.920 ± 0.042 vs 0.881 ± 0.033, P < 0.001) (Table 7.5).

Table 7.5. Mean oxygen uptake (VO_2), heart rate (% of HR_{max}) respiratory exchange ratio (RER) and ratings of perceived exertion (RPE) in the FAST and FED conditions.

Measure	FAST	FED
VO_2 ($L \cdot min^{-1}$)	2.70 ± 0.29	2.71 ± 0.30
HR (% of HR_{max})	79 ± 8	84 ± 5 [c]
RER	0.881 ± 0.033	0.920 ± 0.042 [c]
RPE	12 ± 2	12 ± 1

Values are mean ± 1SD. [c] different (P < 0.001) from FAST.

7.3.6 Glucose, lactate and cortisol

Glucose: There was both a significant main effect of *Time* ($P < 0.001$) and a significant *Group* x *Time* interaction ($P < 0.01$) for blood glucose concentrations. In FAST, blood glucose was not significantly different from BASE before or during exercise, but showed a small but significant decrease ($P < 0.05$) at R3.0 (Figure 7.3, Panel A).

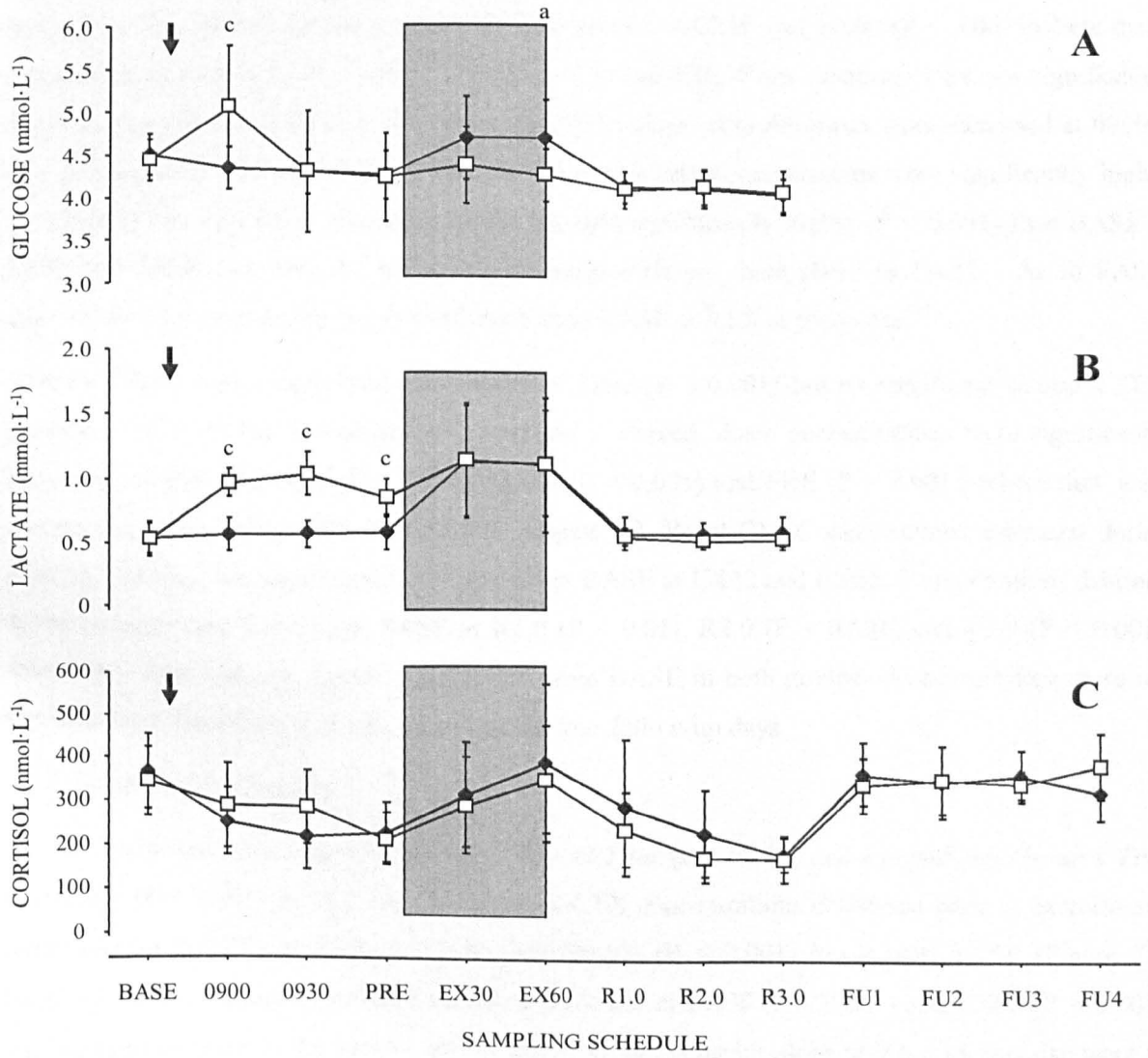

Figure 7.3. Glucose (Panel A), lactate (Panel B) and cortisol (Panel C) concentrations at baseline (BASE), before exercise (0900 - PRE), during exercise (EX30), during 3 h of recovery (EX60 - R3.0) and on 4 follow up days (FU1 – FU4) in the FAST (filled diamonds) and FED (open squares) conditions. Values are mean ± 1SD. Vertical arrow denotes provision of meal in the FED condition. Grey box denotes exercise. Glucose concentrations were significantly lower than BASE at R3.0 only in FAST and significantly higher than BASE at 0900 h only in FED. Lactate concentrations were significantly higher than BASE at EX30 and EX60 in FAST, and from 0900 h up to EX60 in FED. Pooled, cortisol concentrations were significantly lower than BASE from 0900 h up to PRE and again from R1.0 to R3.0. [a] FED different ($P < 0.05$) from FAST; [c] FED different ($P < 0.001$) from FAST.

In FED, glucose was significantly increased ($P < 0.001$) from BASE at 0900 h, and significantly higher ($P < 0.001$) than in FAST. This increase was transient, however, and concentrations were not significantly different from BASE at 9030 h or at any subsequent time point, although they were transiently lower ($P < 0.05$) than in FAST at the end of exercise (Figure 7.3, Panel A).

Lactate: There was both a significant main effect of *Time* ($P < 0.001$) and a significant *Group* x *Time* interaction ($P < 0.001$) for blood lactate concentrations. In FAST, concentrations were unchanged in the pre-exercise period, but were significantly increased at EX30 and EX60 ($P < 0.001$) where mean concentrations were 1.1 ± 0.4 mmol·L^{-1} (Figure 7.3, Panel B). Concentrations were not significantly different from BASE at R1.0 or thereafter. In FED, lactate concentrations were increased at 0900 h ($P < 0.001$), 0930 h ($P < 0.001$) and PRE ($P < 0.001$) where concentrations were significantly higher ($P < 0.001$) than in FAST. Concentrations remained significantly higher ($P < 0.001$) than BASE at EX30 and EX60 but were no longer significantly different from those in FAST. As in FAST, concentrations were not significantly different from BASE at R1.0 or thereafter.

Cortisol: There was a significant main effect of *Time* ($P < 0.001$) but no significant *Group* x *Time* interaction ($P = 0.140$) for cortisol concentrations. Pooled, mean concentrations were significantly lower than BASE at 0900 h ($P < 0.05$), 0930 h ($P < 0.001$) and PRE ($P < 0.001$), where they were decreased approximately 30% from BASE (Figure 7.3, Panel C). Concentrations increased during exercise and were not significantly different from BASE at EX30 and EX60. Concentrations declined thereafter and were lower than BASE at R1.0 ($P < 0.01$), R2.0 ($P < 0.001$) and R3.0 ($P < 0.001$), where they were reduced approximately 45% from BASE in both groups. Concentrations were not significantly different from BASE on any of the four follow-up days.

7.3.7 Bone turnover markers

β-CTX: There was both a significant main effect of *Time* ($P < 0.001$) and a significant *Group* x *Time* interaction ($P < 0.001$) for β-CTX. In FAST, β-CTX concentrations decreased prior to exercise and were reduced by 26% at PRE and were significantly ($P < 0.001$) lower than BASE (Figure 7.4, Panel A). Concentrations remained significantly lower at EX30 ($P < 0.001$) and EX60 ($P < 0.001$), but began to increase in the second half of exercise, and concentrations at 1 h post-exercise were no longer significantly different from BASE. Subsequently, concentrations fell sharply and were significantly lower than BASE at R2.0 ($P < 0.05$) and R3.0 ($P < 0.001$) where they were reduced by 33% from BASE. Concentrations were not significantly different from BASE on any of the four follow-up days. In FED, concentrations were significantly lower than BASE at 0900 h ($P < 0.05$), 0930 h and at PRE ($P < 0.001$), and were significantly lower than FAST at 0930 h and PRE ($P < 0.001$), where the reduction in β-CTX was nearly double that in FAST (47%). Concentrations remained significantly lower than BASE ($P < 0.001$) and FAST ($P < 0.001$) at EX30 and, although they began to increase in the second half of exercise, they remained lower than both BASE ($P < 0.001$)

and FAST ($P < 0.05$) at EX60. By 1 h post-exercise concentrations were no longer significantly different from BASE or FAST.

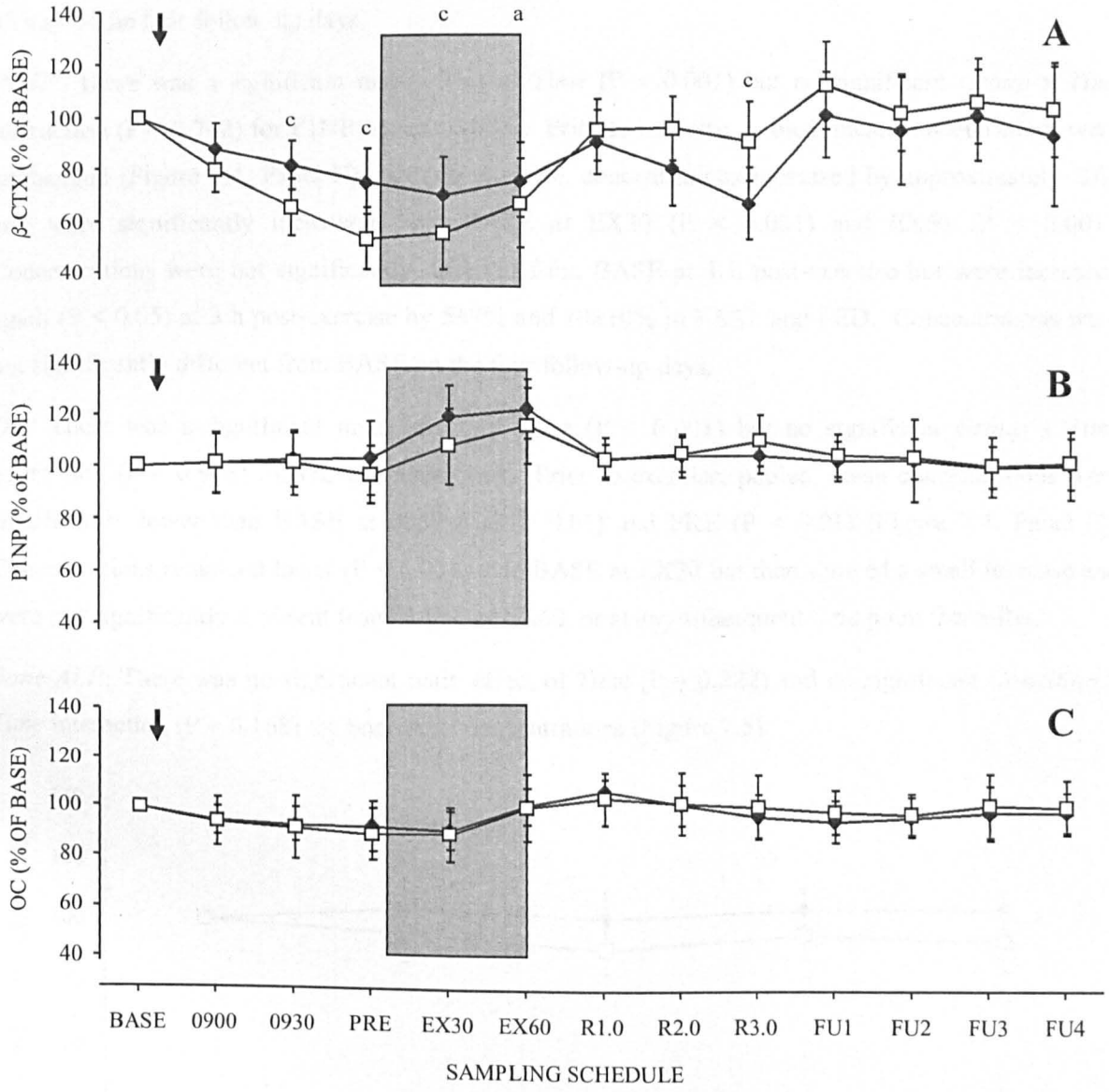

Figure 7.4. β-CTX (Panel A), P1NP (Panel B) and OC (Panel C) concentrations, expressed as a percentage of baseline (BASE), before exercise (0900 h to PRE), during exercise (EX30), during 3 h of recovery (EX60 - R3.0) and 4 follow up days (FU1 – FU4) in the FAST (filled diamonds) and FED (open squares) conditions. Grey box denotes exercise. Vertical arrow denotes provision of meal in the FED condition. Values are mean ± 1SD. β-CTX concentrations in FAST were significantly lower than BASE from PRE up to EX60, and again at R2.0 and R3.0. In FED, concentrations were significantly lower than BASE from 0900 h up to EX60 only. Pooled, P1NP concentrations were significantly higher than BASE at EX30 and EX60, and again at R3.0. Pooled, OC concentrations were significantly lower than BASE at 0930 h and up to PRE only. [a] FED different ($P < 0.05$) from FAST; [c] FED different ($P < 0.001$) from FAST.

Subsequently, however, and unlike in FAST, concentrations only declined by 10% and were not significantly different from BASE at R2.0 and R3.0 and, as a result, were significantly higher ($P < 0.001$) than FAST at R3.0. Concentrations were not significantly different from BASE or FAST on any of the four follow-up days.

P1NP: There was a significant main effect of *Time* ($P < 0.001$) but no significant *Group* x *Time* interaction ($P = 0.742$) for P1NP concentrations. Prior to exercise, pooled, mean concentrations were unchanged (Figure 7.4, Panel B). During exercise, concentrations increased by approximately 20% and were significantly increased from BASE at EX30 ($P < 0.001$) and EX60 ($P < 0.001$). Concentrations were not significantly different from BASE at 1 h post-exercise but were increased again ($P < 0.05$) at 3 h post-exercise by 5±7% and 10±10% in FAST and FED. Concentrations were not significantly different from BASE on the four follow-up days.

OC: There was a significant main effect of *Time* ($P < 0.001$) but no significant *Group* x *Time* interaction ($P = 0.978$) for OC concentrations. Prior to exercise, pooled, mean concentrations were significantly lower than BASE at 0930 h ($P < 0.01$) and PRE ($P < 0.01$) (Figure 7.4, Panel C). Concentrations remained lower ($P < 0.001$) than BASE at EX30 but then showed a small increase and were not significantly different from BASE at EX60, or at any subsequent time point thereafter.

Bone ALP: There was no significant main effect of *Time* ($P = 0.222$) and no significant *Condition* x *Time* interaction ($P = 0.168$) for bone ALP concentrations (Figure 7.5).

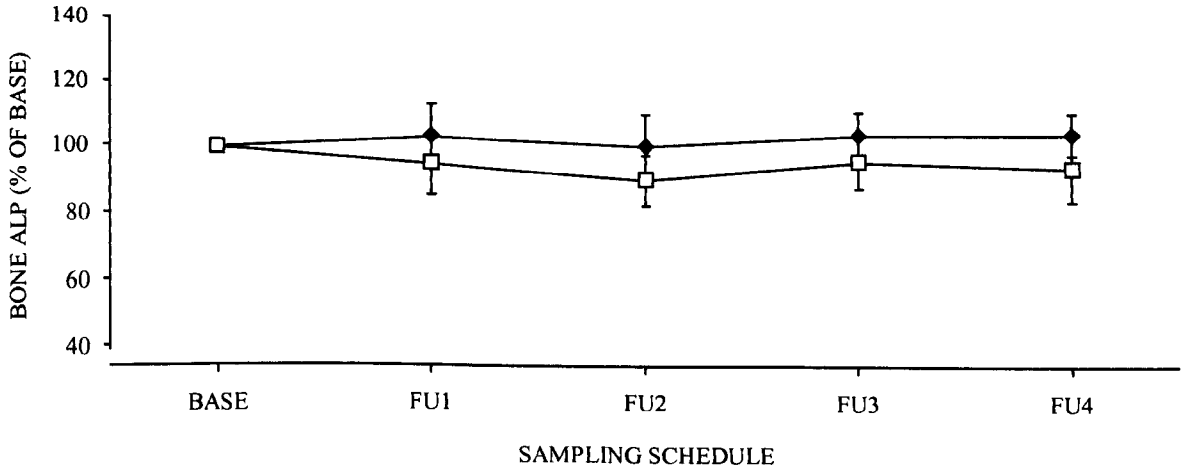

Figure 7.5. Bone ALP concentrations, expressed as a percentage of baseline (BASE) on the four follow up days (FU1 – FU4) in the FAST (filled diamonds) and FED (open squares) conditions. Values are mean ± 1SD.

fPYD/Cr: There was no significant main effect of *Time* ($P = 0.572$) and no significant *Condition* x *Time* interaction ($P = 0.447$) for fPYD/Cr concentrations (Figure 7.6, Panel A).

fDPD/Cr: There was no significant main effect of *Time* ($P = 0.246$) and no significant *Condition* x *Time* interaction ($P = 0.748$) for fDPD/Cr concentrations (Figure 7.6, Panel B).

Cr: There was a significant main effect of *Time* ($P < 0.05$) but no significant *Condition* x *Time* interaction ($P = 0.894$) for Cr concentrations. Pooled, mean Cr concentrations were increased at FU1 but this increase did not reach statistical significance ($P = 0.06$) (Figure 7.6, Panel C).

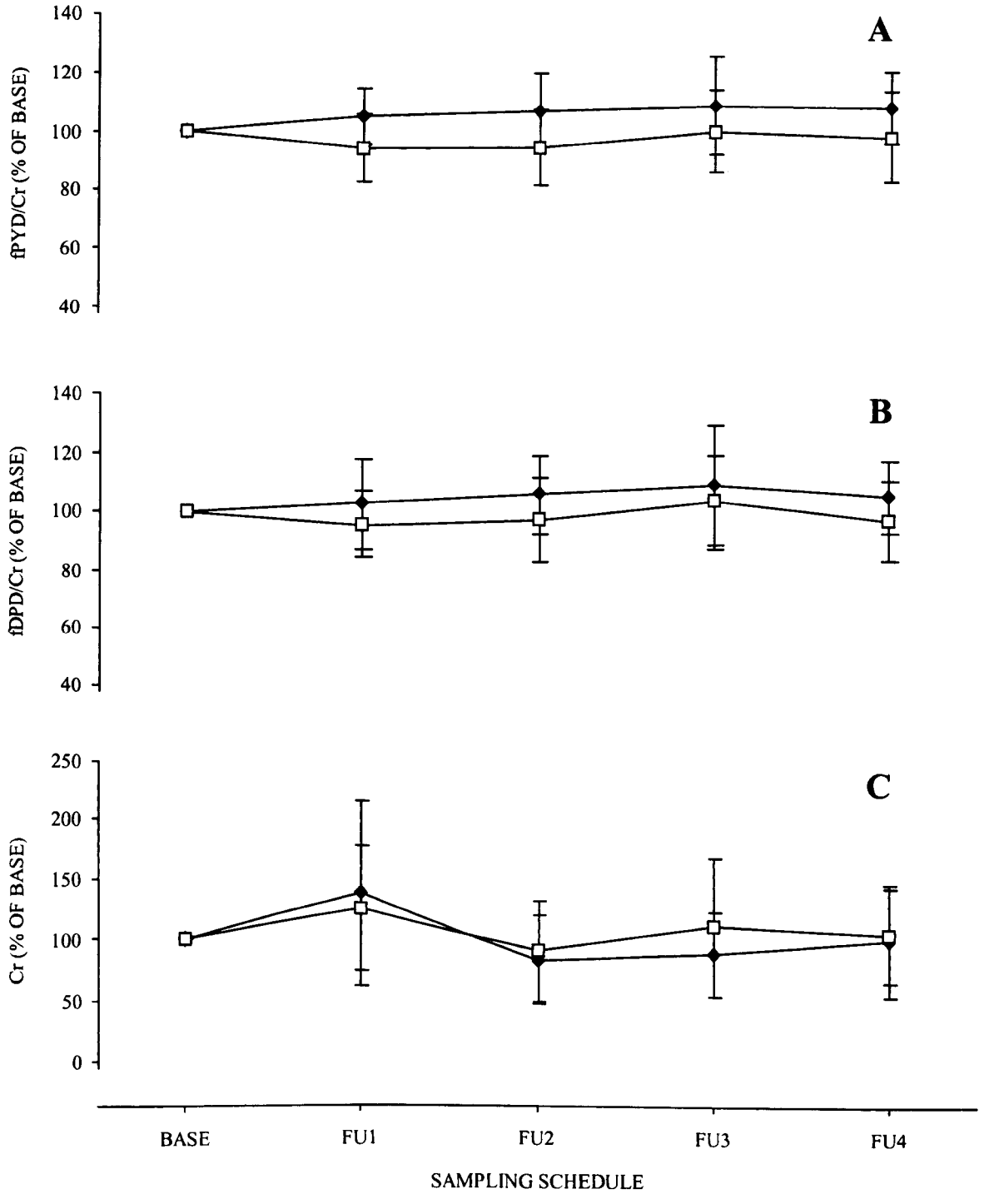

Figure 7.6. The percentage change in BASE concentrations of fPYD/Cr (Panel A), fDPD/Cr (Panel B) and Cr (Panel C) on the four follow-up days (FU1 – FU4) in the FAST (filled diamonds) and FED (open squares) conditions. Values are mean ± 1SD.

Concentrations were not significantly different from BASE at FU2 or thereafter. There was considerable variation in individual Cr response to the two experimental conditions. At FU1, changes in Cr ranged from a 30% decrease up to a 335% increase (Figure 7.7, Panels A and B). On the subsequent follow-up days, increases were less pronounced (maximum = 214% of BASE) although several subjects displayed larger decreases (minimum = 20% of BASE). Some subjects displayed both considerable increases and decreases on consecutive follow-up days in the same condition.

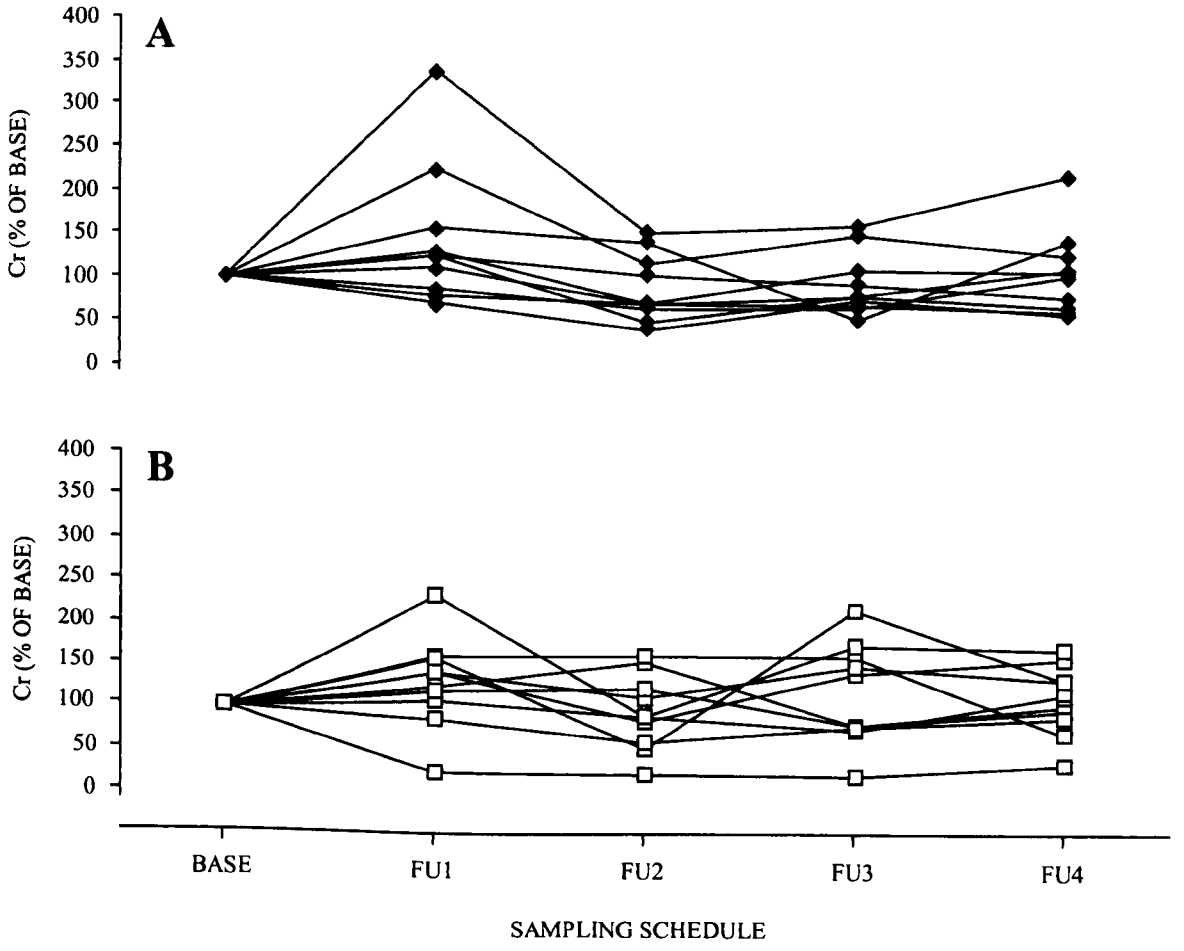

Figure 7.7. Individual changes in Cr, expressed as the percentage change in BASE concentrations on the four follow-up days (FU1 – FU4) in the FAST (Panel A) and FED (Panel B) conditions.

fPYD output: There was a significant main effect of *Time* ($P < 0.05$) but no significant *Condition* x *Time* interaction ($P = 0.593$) for fPYD output. Pooled, mean output was not significantly different from BASE at FU1, FU2 or FU3 but showed a small but significant ($P < 0.01$) increase at FU4 (Figure 7.8, Panel A).

fDPD output: There was a significant main effect of *Time* ($P < 0.035$) but no significant *Condition* x *Time* interaction ($P = 0.574$) for fDPD output. Pooled, mean output was not significantly different

from BASE at FU1, FU2 or FU3 but showed a small but significant increase ($P < 0.01$) at FU4 (Figure 7.8, Panel B).

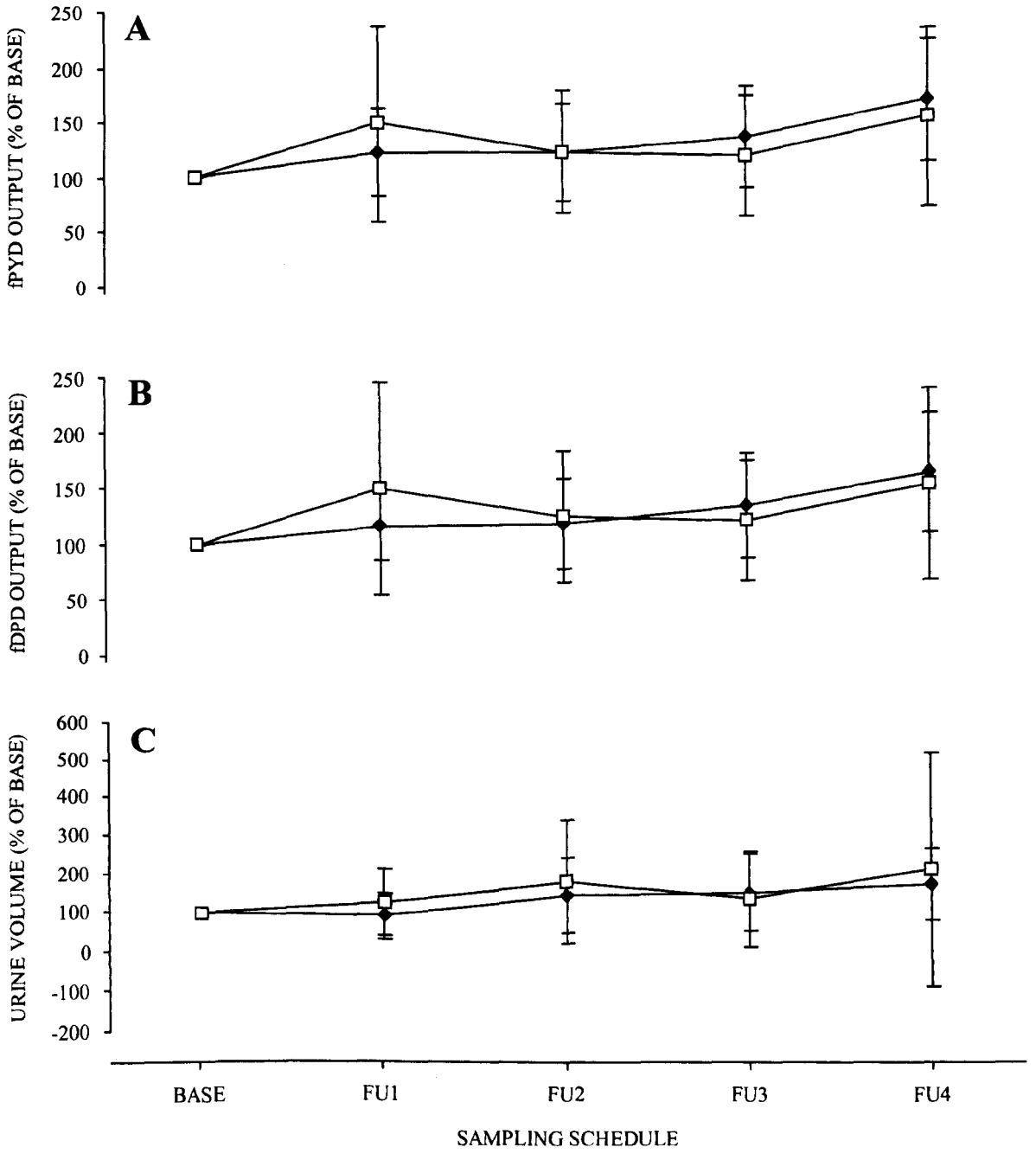

Figure 7.8. The percentage change from BASE of fPYD (Panel A) and fDPD (Panel B) output, and urine volume (Panel C), on the four follow-up days (FU1 – FU4) in the FAST (filled diamonds) and FED (open squares) conditions. Values are mean ± 1SD. fPYD or fDPD output (mmol) = fPYD or fDPD ($mmol \cdot L^{-1}$) x urine volume (L). Pooled, fPYD output and fDPD output were significantly increased from BASE at FU4 only.

Urine volume: There was no significant main effect of *Time* (P = 0.165) and no significant *Condition* x *Time* interaction (P = 0.754) for urine volume (Figure 7.8, Panel C). There was considerable variability in BASE urine volume between individual subjects (0.028 to 0.207 L). This variability increased in samples across all five sampling points (0.027 L and 0.478 L). There was no consistent pattern of change from BASE across the four follow-up days with both increases and decreases in the volume of urine produced compared to that at BASE (Figure 7.9, Panels A and B). There was also variability within individual subjects across four consecutive follow-up days. For example, at FU2, one subject produced 229% more urine than at BASE but 50% less at FU4. Another subject produced similar volumes to BASE at FU2 and FU4 but 345% more than BASE at FU3.

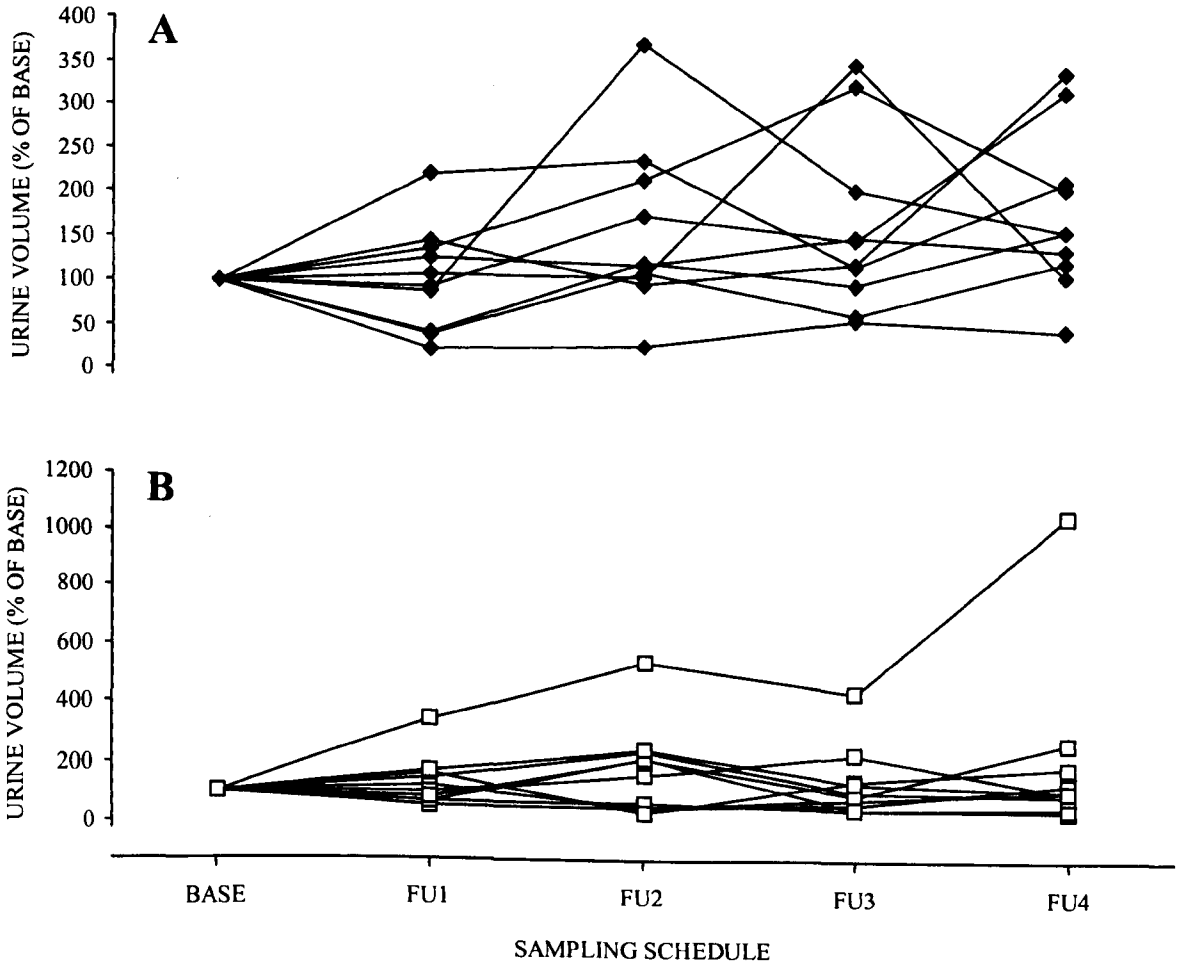

Figure 7.9. Individual changes in second void urine volume, expressed as the percentage change in BASE concentrations, on the four follow-up days (FU1 – FU4) in the FAST (Panel A) and FED (Panel B) conditions. Different scales are used for clarity.

7.3.8 OPG

There was a significant main effect of *Time* ($P < 0.001$) but no significant *Condition* x *Time* interaction ($P = 0.403$) for OPG concentrations. Pooled, mean OPG concentrations were unchanged at 0900 h and 0930 h, but by PRE, concentrations were significantly ($P < 0.01$) increased (33 ± 24% in FAST and 11 ± 24% in FED) compared with BASE (Figure 7.10). OPG remained significantly increased ($P < 0.001$) from BASE throughout exercise and in the first 3 h of recovery, with peak concentrations occurring at EX30 in FAST and EX60 in FED where concentrations were increased by 40%. Concentrations were not significantly different from BASE at FU1 or thereafter.

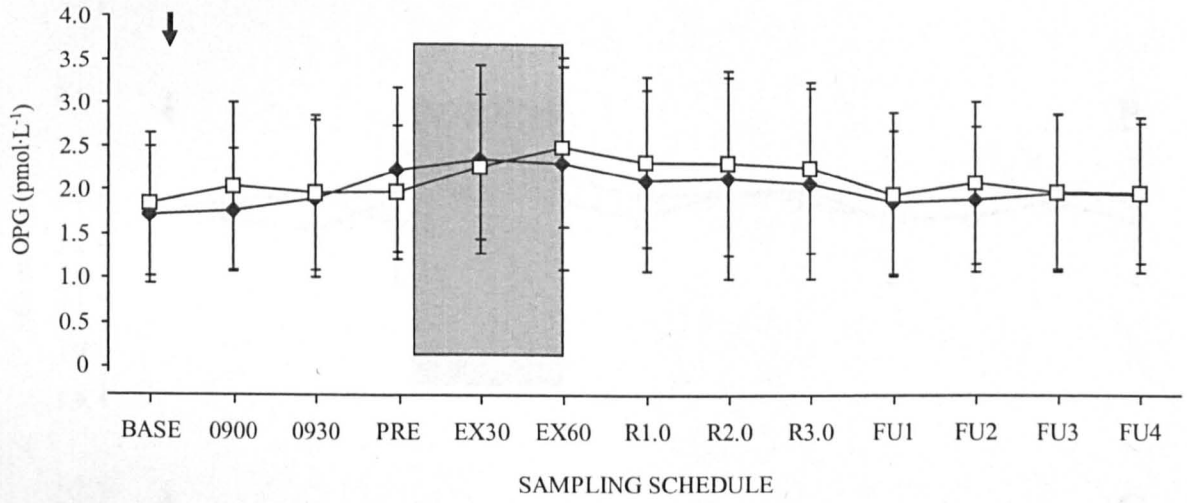

Figure 7.10. OPG concentrations at baseline (BASE), before exercise (0900 h - PRE), during exercise (EX30), during 3 h of recovery (EX60 - R3.0) and 4 follow up days (FU1 – FU4) in the FAST (filled diamonds) and FED (open squares) conditions. Vertical arrow denotes provision of meal in the FED condition. Grey box denotes exercise. Values are mean ± 1SD. Pooled, OPG concentrations were significantly increased from BASE at PRE and up to R3.0.

7.3.9 Calcium Metabolism

PTH: There was a significant main effect of *Time* ($P < 0.001$) but no significant *Condition* x *Time* interaction ($P = 0.130$) for PTH concentrations. Pooled, mean PTH concentrations were significantly ($P < 0.05$) lower than BASE at 0900 h but not at 0930 h or PRE (Figure 7.11, Panel A). PTH increased during exercise resulting in significantly higher concentrations at EX30 ($P < 0.001$) and at EX60 ($P < 0.001$) compared with BASE, where concentrations were increased by 87% and 71% in FAST and FED. PTH concentrations were not different from BASE at R1.0 and remained so up to 3 h of recovery. PTH was significantly higher ($P < 0.05$) than BASE at FU1 but not thereafter.

ACa: There was no significant main effect of *Time* (P = 0.387) and no significant *Condition* x *Time* interaction (P = 0.450) for ACa concentrations (Figure 7.11, Panel B).

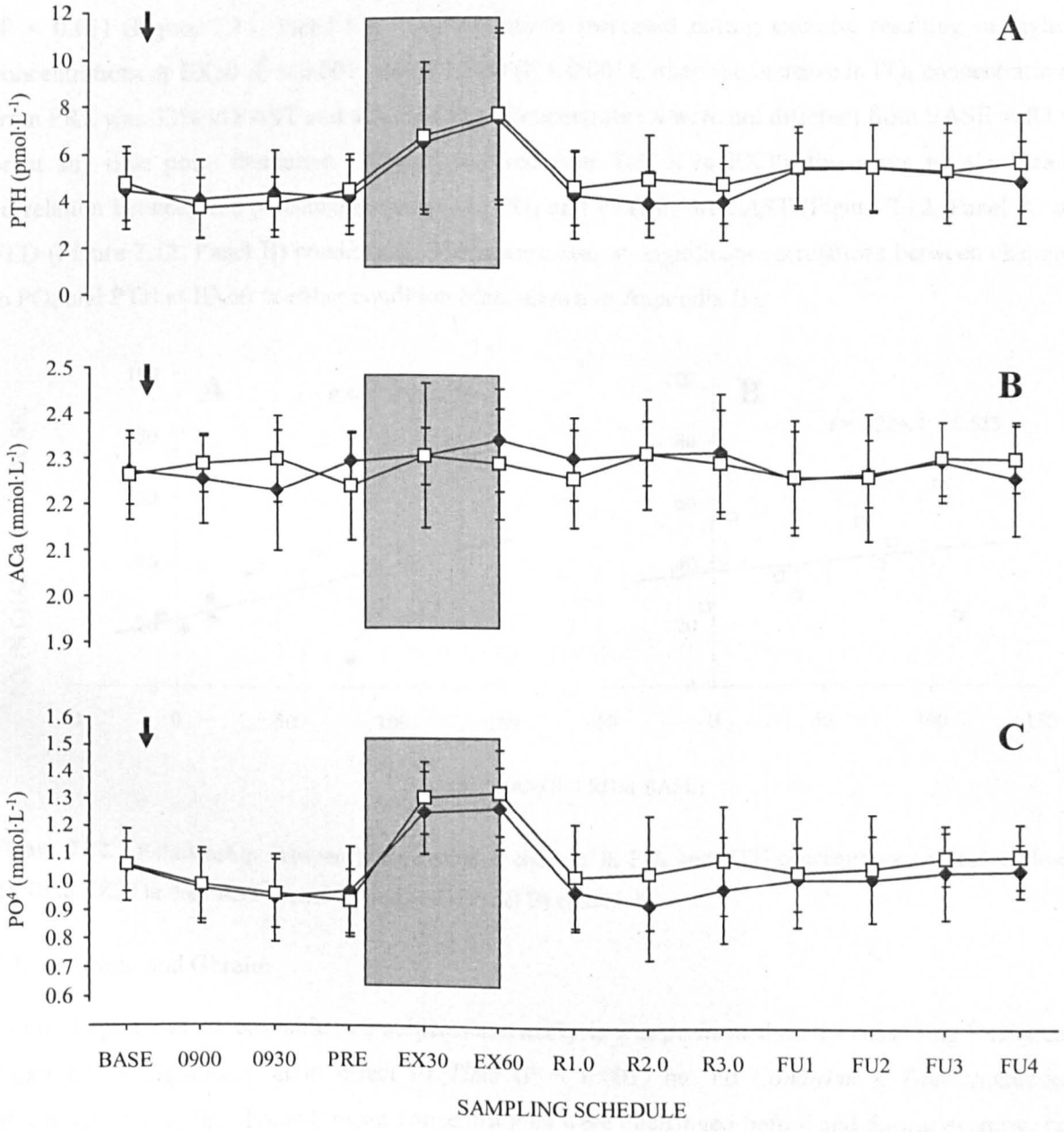

Figure 7.11. PTH (Panel A), ACa (Panel B) and PO_4 (Panel C) concentrations at baseline (BASE), before exercise (0900 h - PRE), during exercise (EX30), during 3 h of recovery (EX60 - R3.0) and 4 follow up days (FU1 – FU4) in the FAST (filled diamonds) and FED (open squares) conditions. Vertical arrow denotes provision of meal in the FED condition. Grey box denotes exercise. Values are mean ± 1SD. Pooled, PTH concentrations were significantly lower than BASE at 0900 h, significantly increased at EX30 and EX60, and again at FU1. Pooled PO_4 concentrations were significantly lower than BASE at 0930 h and PRE, and significantly increased at EX30 and EX60.

PO_4: There was a significant main effect of *Time* ($P < 0.001$) but no significant *Condition* x *Time* interaction ($P = 0.680$) for PO_4 concentrations. Pooled, mean PO_4 concentrations declined in the pre-exercise period and were approximately 10% lower than BASE at 0930 h ($P < 0.01$) and PRE ($P < 0.01$) (Figure 7.11, Panel C). Concentrations increased during exercise resulting in higher concentrations at EX30 ($P < 0.001$) and at EX60 ($P < 0.001$), when the increase in PO_4 concentrations from PRE was 33% in FAST and 42% in FED. Concentrations were not different from BASE at R1.0 or at any time point thereafter. When measured from BASE to EX30, there was no significant correlation between the percentage changes in PO_4 and PTH in the FAST (Figure 7.12, Panel A) or FED (Figure 7.12, Panel B) conditions. There were also no significant correlations between changes in PO_4 and PTH at EX60 in either condition (data shown in Appendix B).

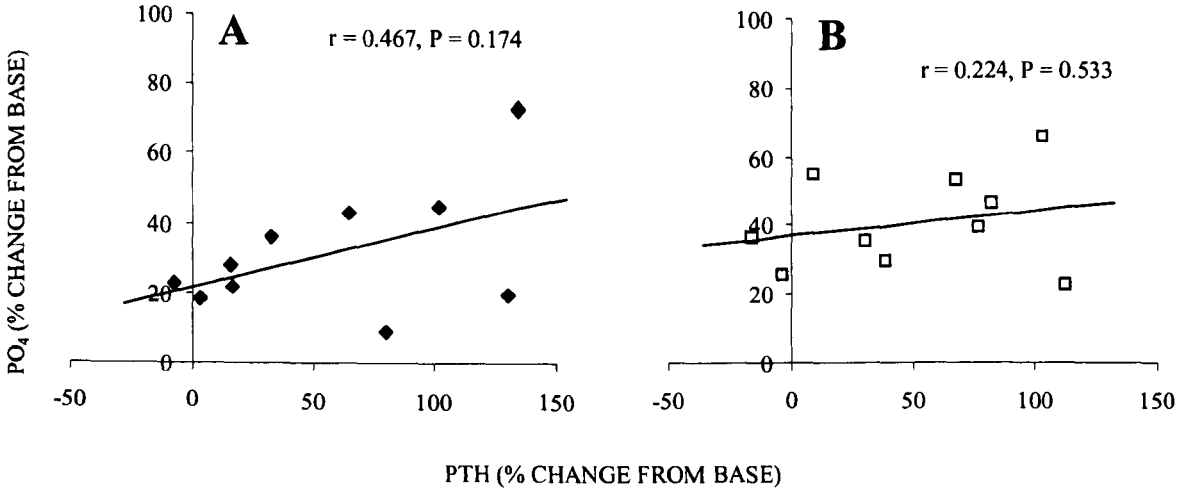

Figure 7.12. Relationship between the percentage changes in PO_4 and PTH concentrations measured from BASE to EX30 in the FAST (Panel A) and FED (Panel B) conditions.

7.3.10 Leptin and Ghrelin

Leptin: Leptin was undetectable in 3 subjects and analysis was performed on the remaining 7 subjects. There was a significant main effect of *Time* ($P < 0.001$) but no *Condition* x *Time* interaction ($P = 0.520$) for leptin. Pooled, mean concentrations were unchanged before and during exercise, but were significantly lower than baseline at 1 h ($P < 0.01$), 2 h ($P < 0.05$) and 3 h ($P < 0.01$) post-exercise (Figure 7.13, Panel A). Concentrations were not significantly different from baseline in either group from 1 to 4 days post-exercise.

Ghrelin: There was a significant main effect of *Time* ($P < 0.001$) but no *Condition* x *Time* interaction ($P = 0.158$) for ghrelin. Despite the significant main effect of *Time*, pooled, mean concentrations were not significantly different from baseline at any subsequent time point (Figure 7.13, Panel B).

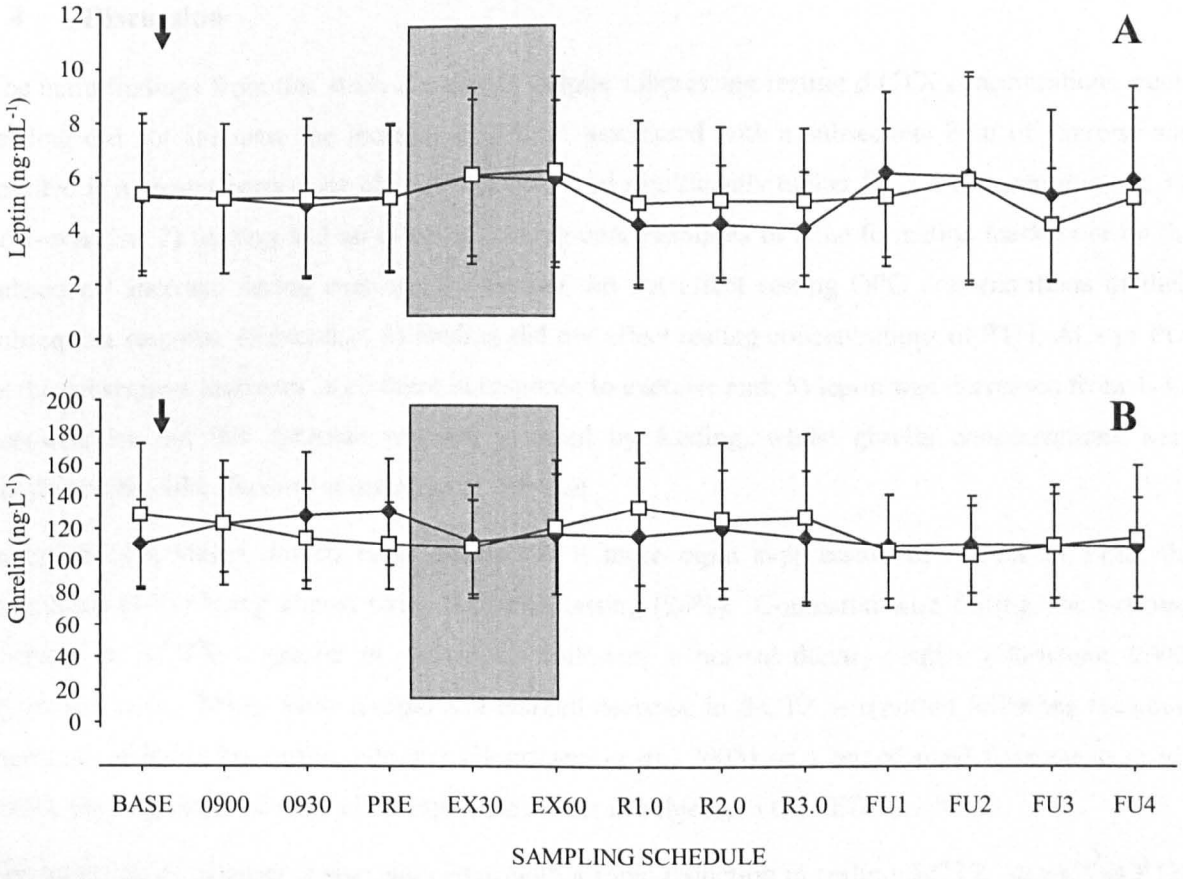

Figure 7.13. Leptin (Panel A) and ghrelin (Panel B) concentrations at baseline (BASE), before exercise (0900 - PRE), during exercise (EX30), during 3 h of recovery (EX60 - R3.0) and 4 follow up days (FU1 – FU4) in the FAST (filled diamonds) and FED (open squares) conditions. Values are mean ± 1SD. Vertical arrow denotes provision of meal in the FED condition. Grey box denotes exercise. Leptin was undetectable in 3 subjects and analysis was performed on the remaining subjects (n=7). Pooled, leptin concentrations were significantly lower than baseline from R1.0 to R3.0 only.

7.4 Discussion

The main findings from this study are that 1) despite suppressing resting β-CTX concentrations, acute feeding did not attenuate the increase in β-CTX associated with a subsequent bout of exercise and resulted in a greater percentage change in β-CTX and significantly higher β-CTX concentrations at 3 h post-exercise; 2) feeding had no effect on resting concentrations of bone formation markers or on the subsequent increase during exercise; 3) feeding did not affect resting OPG concentrations or their subsequent response to exercise; 4) feeding did not affect resting concentrations of PTH, ACa or PO_4 or the subsequent increases in all three in response to exercise and; 5) leptin was decreased from 1-3 h post-exercise but this decrease was not affected by feeding, whilst ghrelin concentrations were unaffected by either feeding or subsequent exercise.

Ingestion of a single, mixed meal resulted in a more rapid suppression of β-CTX in FED, the magnitude (47%) being almost twice that with fasting (26%). Compared with fasting, the morning decrease in β-CTX is greater in individuals following a normal dietary routine (Christgau, 2000; Bjarnason *et al.*, 2002), while a rapid and marked decrease in β-CTX is reported following the acute ingestion of individual macronutrients (Henriksen *et al.*, 2003) or a mixed meal (Bjarnason *et al.*, 2002), the magnitude of which is comparable to that in subjects in the FED condition.

The ingestion of calcium is also associated with a rapid reduction in resting β-CTX, as well as PTH, with the change in the later likely mediating that in β-CTX (Guillemant *et al.*, 2000; Zikán *et al.*, 2001). As the meal ingested in the present study contained 116 mg of calcium, an effect of calcium ingestion on β-CTX cannot be excluded completely. However, as 116 mg is only approximately one third of that shown to suppress both PTH and β-CTX (Guillemant *et al.*, 2000), and there was no effect of feeding on PTH prior to exercise, calcium is unlikely to have had a significant effect. Indeed, the decrease in β-CTX following macronutrient ingestion is unlikely to be explained by decreased PTH (Clowes *et al.*, 2002b) but might be related to changes in pancreatic or enteric hormones, although this mechanism remains to be fully described (Henriksen *et al.*, 2003; Clowes *et al.*, 2005).

It was hypothesised that the suppressive effect of food ingestion on β-CTX would be effective in attenuating the increase in β-CTX reported with acute endurance exercise (Guillemant *et al.*, 2004; Maïmoun *et al.*, 2006). Contrary to the hypothesis, and despite the suppression of resting β-CTX by feeding, concentrations subsequently increased during exercise resulting in similar β-CTX concentrations in the FED and FAST groups 1 h post-exercise.

The increase in β-CTX in the second half of exercise that peaked at 1 h post exercise in both conditions is similar to that reported by Guillemant *et al.* (2004). When measured from immediately pre-exercise, to 1 h post-exercise, Guillemant *et al.* (2004) report a 45-50% increase in β-CTX in pre-fed subjects. Over the same time period in Study III, β-CTX increased only 3% with running at 75% VO_{2max} in fasted subjects, and at 65% VO_{2max}, was decreased by 10%. In the present study, over

an identical time frame, β-CTX increased 86% in FED and 22% in FAST subjects with 60 min of running at only 65% VO_{2max}. However, when compared with blood samples taken at baseline, 2.5 h prior to exercise, β-CTX concentrations at R1.0 were not significantly different from baseline in either FAST or FED. This suggests that pre-exercise nutritional status and the timing of the 'baseline' sample are important factors in the interpretation of the effect of acute, endurance exercise on β-CTX concentrations when performed in the late morning. In addition, as the only difference in the study protocols between the MOD condition in Study III and the FAST condition in the present study was the start time of exercise (1015 h vs 0815 h), this suggests that the timing of exercise itself also influences the β-CTX response.

Compared with no calcium supplementation, Guillemant *et al.* (2004) showed ingestion of 972 mg of calcium before and during exercise completely suppressed the exercise-associated increase in β-CTX. The success of calcium to suppress the increase in β-CTX in the study of Guillemant *et al.* (2004) and the failure of a mixed meal to do so in the present study is not fully explained by differences in their potency to suppress β-CTX, as the speed (within 1 h) and magnitude (50%) of suppression with food (Bjarnason *et al.*, 2002) is comparable to that with 344 mg calcium (Guillemant *et al.*, 2000).

Guillemant *et al.* (2004) attribute the effect of calcium ingestion on β-CTX to the smaller increase in PTH concentrations during exercise with calcium (70%), compared to the no calcium trial (250-300% increase). As in the study of Guillemant *et al.* (2004), the increase in PTH in the present study occurred prior to the increase in β-CTX, suggesting that changes in PTH might be mediating the β-CTX response. The relative increase in PTH in FAST and FED (70-90%) is similar to that reported by Guillemant *et al.* (2004) with calcium ingestion where there was no increase in β-CTX. However, peak concentrations in the present study (7.5 $pmol \cdot L^{-1}$) were considerably higher than those reported by Guillemant *et al.* (2004) with calcium ingestion (36 $pg \cdot mL^{-1}$ or 3.8 $pmol \cdot L^{-1}$), and higher than even without calcium (63 $pg \cdot mL^{-1}$ or 6.6 $pmol \cdot L^{-1}$), suggesting that the absolute PTH concentration during exercise maybe a factor in determining subsequent changes in β-CTX.

Finally, the different effects on β-CTX in the study of Guillemant *et al.* (2004) and the present study might be explained by the different ingestion schedules, as it has recently been shown that the acute ingestion of calcium before exercise only, does not affect the subsequent β-CTX response (Barry *et al.*, 2009). Therefore, the timing of 'nutritional' interventions might determine their effect on bone resorption, and ingestion both before and during exercise might be required to attenuate the β-CTX response.

An unexpected finding of the present study was the different β-CTX response in FED compared with FAST in the early post-exercise period. The significant reduction in β-CTX to 80% and 67% of BASE values at 2 h and 3 h post-exercise in FAST is consistent with its circadian rhythm and the timing (from 1 h post-exercise) and magnitude (30%) of the decrease is comparable to that observed in Study

III. Taken together, these results suggest that, despite an effect of this type of exercise in increasing β-CTX, its entrained circadian rhythm remains a strong influence on the circulating concentrations. In contrast, no significant decrease was seen in FED over the same period suggesting that feeding prior to exercise alters the entrained rhythm of β-CTX. However, the magnitude of this effect was relatively modest.

The differences in post-exercise β-CTX concentrations are not explained by differences in exercise intensity as O_2 uptake and blood lactate levels were not significantly different between the two conditions, while the lower heart rate with pre-feeding is consistent with previous studies (Nieman *et al.*, 1987), as is the lower RER (Coyle *et al.*, 1985). The difference is also not explained by differences in PTH responses, as the magnitude and duration of the increase in PTH to exercise was similar in both conditions. Unchanged resting leptin concentrations following the ingestion of a meal in the present study is consistent with some (Korbonits *et al.*, 1997; Erdmann *et al.*, 2005; Kalaitzakis *et al.*, 2007) but not all (Dallongeville *et al.*, 1998; Poppitt *et al.*, 2006) studies of acute feeding. Although the quantitative reduction (10%) in ghrelin at 2 h post-feeding in FED is similar to that reported by Callahan *et al.*, (2004) following a meal of similar calorie load, there was no significant effect of feeding on ghrelin which would be consistent with ghrelin not being involved in the short-term physiological control of bone resorption around feeding (Huda *et al.*, 2007). The failure of exercise to affect ghrelin concentrations is consistent with previous studies (Dall *et al.*, 2002; Burns *et al.*, 2007).

The significant decrease in leptin from 1-3 h post-exercise is unlikely to be related to exercise alone as previous studies indicate that leptin concentrations are only reduced following long duration, strenuous exercise (Landt *et al.*, 1997; Racette *et al.*, 1997; Dulcos *et al.*, 1999; Olive and Miller, 2001; Keller *et al.*, 2005b), with a marked reduction evident immediately after exercise. In the study of Keller *et al.*, (2005b) leptin levels also decreased in the resting control group by 25% from 0800 h to 1330 h, possibly as a result of its circadian rhythm (Gavrila *et al.*, 2003), which might explain the reduced post-exercise concentrations in FAST and FED. Pre-feeding is reported to increase the leptin response to a VO_{2max} test (Sliwowski *et al.*, 2001) but have no effect on leptin or ghrelin levels during 15 min of incremental work (Zoladz *et al.*, 2005). The failure of feeding to affect leptin or ghrelin concentrations before, during or after exercise in the present study suggests no role for either in the suppression of resting β-CTX prior to exercise or the different responses in the early post-exercise period.

Several other hormones released during the normal physiological response to nutrient ingestion potentially integrate nutrient intake and bone turnover. Blood glucose was transiently increased following feeding but, *in vivo*, the hyperinsulinemia associated with hyperglycaemia following feeding has a relatively small effect on bone turnover (Clowes *et al.*, 2002b). The gastrointestinal hormones glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide (GLP)-1 and GLP-2 are

secreted following feeding (Elliot *et al.*, 1993; Henriksen *et al.*, 2003). Only GLP-2 has been shown to independently suppress β-CTX concentrations although the effect requires an increase in circulating concentrations far in excess of that observed with feeding (Henriksen *et al.*, 2003), while the effect of acute exercise on GLP-2 remains unknown. The relatively slow effects of feeding on epinephrine, norepinephrine and growth hormone (Tse *et al.*, 1983a; 1983b) are unlikely to mediate the rapid decrease in β-CTX with feeding alone. However, as they are all known to increase in response to acute exercise, an interaction with feeding, exercise and bone turnover cannot be excluded. Thus, it is possible that a yet unidentified factor, modulated by feeding, or the combination of feeding and subsequent exercise, acts to sustain the increase in β-CTX associated with acute exercise.

Resting concentrations of P1NP and OC were unchanged with acute feeding and only showed modest changes with subsequent exercise, whilst bone ALP was unaffected by either condition when measured from 1 to 4 days post-exercise. The lack of any effect of acute feeding on OC is consistent with previous studies (Bjarnason *et al.*, 2002; Henriksen *et al.*, 2003), although other studies report a small (<10%) but significant lowering of OC concentrations following the ingestion of glucose (Clowes *et al.*, 2003) or a mixed meal (Clowes *et al.*, 2002a). Decreases in P1NP of less than 10% have been reported following a mixed meal (Clowes *et al.*, 2002a) and glucose ingestion (Clowes *et al.*, 2003). This might, in part, explain why P1NP concentrations in the present study tended to be 5-10% lower in FED immediately before and throughout exercise.

The 15-20% increase in P1NP seen with exercise in FAST and FED is comparable with the 14% increase in the MOD condition in Study III and confirms a rapid but transient increase with acute running. This response is in contrast to existing studies (Tosun *et al.*, 2006; Pomerants *et al.*, 2008) although the small increase in P1NP at 3 h post-exercise is in agreement with a previous study of cycling by Herrmann *et al.* (2007). As in Study III, whether the transient increase in P1NP during exercise represents an increase in type 1 collagen formation in bone is unclear, particularly as it precedes an increase in β-CTX. Again, changes in blood flow with exercise and a contribution to the circulating P1NP concentration from other tissues containing type 1 collagen (*e.g.* skeletal muscle, tendon) cannot be excluded (Crameri *et al.*, 2004; Miller *et al.*, 2007; Hansen *et al.*, 2008).

The significant decrease in OC concentrations in the first half of exercise might reflect its circadian rhythm (Nielsen *et al.*, 1990). Therefore, as in Study III, the return of OC concentrations to baseline values at the end of exercise likely represents a genuine effect of exercise. Declining basal concentrations and only a modest increase with exercise might explain why some studies of moderate intensity endurance exercise report no increase in OC (Rudberg *et al.*, 2000; Tosun *et al.*, 2006). Again, as in Study III, the increase in OC occurred despite an increase in cortisol concentrations suggesting that, unlike at rest, during exercise OC concentrations are not regulated by changes in cortisol. Taken together, the changes in P1NP, OC and bone ALP suggest that, at least up to 4 days

post-exercise, 60 min of running at 65% VO_{2max} has only a modest and transient effect on bone formation, evident only during exercise itself.

Combined with modest changes in bone formation markers, the failure of acute feeding to suppress the increase in β-CTX with subsequent exercise suggests that it does not favourably modulate the negative bone remodelling balance reported previously during acute endurance exercise (Guillemant *et al.*, 2004). Although the higher post-exercise β-CTX concentrations suggest a less favourable response to exercise with feeding, due to the transient nature of this difference, its significance is unclear, particularly as prior to exercise, β-CTX was suppressed by a greater amount compared with fasting. The failure of feeding to suppress the acute β-CTX response to exercise and the lack of any changes in β-CTX on the four recovery days also suggests that the difference in pre-exercise nutritional status of subjects in Study I and Study II is unlikely to explain the different β-CTX responses seen in these studies.

There was no effect of either protocol on fPYD/Cr or fDPD/Cr when measured on the four recovery days. As β-CTX was increased only transiently with exercise in both conditions, it is possible that this effect was not of sufficient magnitude to be evident in fPYD and fDPD in a SMV urine sample the following morning. Alternatively, as there was a tendency for increased Cr concentrations at FU1, it is also possible that any effect of exercise on pyridinolines was masked when concentrations were corrected for Cr. As in Study III, exercise was associated with both increased and decreased Cr concentrations in individual subjects on the day after exercise. The variability of this response can be demonstrated by looking at several individual subjects. Although there was no difference between mean Cr concentrations between FAST and FED, in one subject Cr was increased more than 3-fold from BASE at FU1 in FAST, but was reduced to only 25% of BASE levels in FED at the same time point. In another subject, in FAST, Cr was increased 23% at FU1 but decreased by 52% at FU2. Similarly in FED, in one subject Cr was increased 58% at FU1 but decreased by 48% at FU2. In three subjects in FAST and two in FED, despite the tendency for increased mean Cr concentrations, Cr was below BASE levels on all four follow-up days. Whilst the general effect of exercise in increasing Cr concentrations has implications for its use as a corrective factor for urinary bone markers, the variability in Cr responses (both above and below baseline) and their magnitude is likely to be equally problematic.

In contrast to Cr-corrected pyridinolines, fPYD and fDPD output was significantly increased at FU4. With no change from BASE in mean urine volume on the four follow-up days, this might indicate a genuine increase in pyridinolines and not an artefact of a systematic increase in urine volume. However, like in Studies II and III, the lack of change in mean urine volume masks considerable variations in volume within individual subjects. Importantly, at FU4, urine volume was increased by more than 50% in 7 subjects in FAST but decreased in the two others, while in FED, a greater than 50% increase was seen in 4 subjects but urine volume was decreased in three others. Thus, as in Study

III, consistent mean urine volumes in consecutive SMV samples are not indicative of the considerable day-to-day variation within individuals. Compared with Study II where pyridinoline output was increased at FU4, the effect of exercise in the present study at the same time point was the opposite. Although two bouts of exercise were performed in Study II, given the lack of change in β-CTX at FU4 in the present study, this raises further questions regarding the usefulness of free pyridinoline output in SMV urine samples as a measure of bone resorption under these conditions.

In the 2 h prior to exercise, OPG concentrations were not affected by acute feeding despite a marked reduction in β-CTX. Chailurkit *et al.* (2008) report OPG concentrations to be 10% lower following acute glucose ingestion, although this effect appears to be related to the post-feeding hyperinsulinemia rather than hyperglycaemia itself (Knudsen *et al.*, 2007). This might make glucose a more potent suppressor of OPG concentrations compared with a mixed meal due to a more marked increase in insulin concentrations with glucose. Alternatively, as OPG was increased 33% in FAST compared to only 11% in FED by pre-exercise, this might suggest that a longer period of analysis without exercise might have revealed an attenuation of the morning increase in OPG with feeding, with changes in OPG lagging those of β-CTX. That said, however, the maximal negative correlation between OPG and β-CTX in circadian rhythm studies occurs when changes in OPG precede those in β-CTX, rather than vice versa (Joseph *et al.*, 2007).

As OPG was significantly increased from BASE prior to exercise (up to 1015 h), this might suggest that at least some of the increase in OPG seen during exercise in Studies I and III might be due to its circadian rhythm (Joseph *et al.*, 2007). The lack of any further increase in OPG by the end of exercise in the FAST condition lends some support to this idea. In contrast, however in FED, OPG concentrations increased by a further 30% by the end of exercise. Although there was no significant difference in the overall pattern of change in OPG between the FAST and FED conditions, the small differences described above suggest that the interactions between feeding and exercise on OPG might warrant further investigation.

As in the pre-exercise period, despite different β-CTX responses at 2 h and 3 h post-exercise in FAST and FED, there were no significant differences in OPG concentrations. Taken together, these findings suggest that, under these conditions, acute changes in OPG concentrations do not reflect changes in β-CTX. Given the relationship between RANKL, RANK and OPG in the control of bone turnover, a reduction in RANKL would also serve to reduce bone resorption activity. Thus, a change in the ratio of RANKL to OPG, rather than a change in OPG alone, might, in part, be involved in the changes in β-CTX seen in the present study.

The only previous study to examine the effects of a mixed meal on PTH report increased PTH following feeding (Sethi *et al.*, 1983). Subsequently, however, PTH is reported to decrease following glucose ingestion (D'Erasmo *et al.*, 1999; Clowes *et al.*, 2003) and induced hyperinsulinemia

(Nowicki *et al.*, 1998; Clowes *et al.*, 2002b). In the present study, PTH concentrations were reduced by 22% in FED compared with only 9% in FAST at 0900 h. The decrease in PTH in the study of Clowes *et al.* (2003) occurred 20 min after glucose ingestion but was no longer evident at 40 min. Thus, by only sampling at 0900 h in the present study, 45 min after the meal was ingested, it is possible that the point of maximal suppression of PTH by feeding was missed.

The increase in PTH during subsequent running was transient, peaking at the termination of exercise, with the magnitude of the increase unaffected by the prior ingestion of food. This profile of change is similar to that produced with daily PTH injections (Qin *et al.*, 2004) that increase bone formation and bone mass (Neer *et al.*, 2001). However, apart from the modest and transient increases in OC and P1NP during exercise, as in Studies I, II and III, the increase in PTH was not associated with a marked or prolonged increase in bone formation markers.

The increase in PTH did, however, precede the increase in β-CTX, as has been reported in circadian rhythm studies (Joseph *et al.*, 2007). As there was a clear increase in β-CTX, this perhaps provides more convincing evidence than does Study III that the increase is related to the proceeding increase in PTH. Again, the apparent shorter 'lag' time (~1 h) between changes in PTH and β-CTX compared to that in the circadian rhythms (2 h) might be due to a more marked and rapid increase in PTH. As in Study III, in FAST the decrease in PTH concentrations with the termination of exercise preceded the decrease in β-CTX concentrations by approximately 1 h, further suggesting an association between the two. In FED, however despite a similar pattern of change in PTH to that in FAST, there was no significant decrease in β-CTX at 2 and 3 h post-exercise. This finding appears to question the strength of any association between PTH and bone resorption under these conditions.

Although repeated, transient increases in PTH are associated with increased bone formation, a single injection of PTH 1-34 increases PTH concentrations within 30 min and β-CTX within 60 min (Zikán and Stepan, 2008), suggesting that a period of bone resorption may actually be a necessary pre-requisite for the anabolic effects of PTH. Therefore, although the results of the present study suggest no effect of exercise on bone formation despite a transient increase in PTH, it is possible that the increase in β-CTX was in fact the early phase of a longer-term anabolic effect that went undetected due to a follow-up period of only four days.

In contrast to Studies I, II and III, in the present study there was no significant decrease in PTH in the early post-exercise period. Although there was no significant difference in post-exercise PTH concentrations between the two groups, PTH concentrations were 10% lower than BASE at 2 h and 3 h post-exercise in FAST whereas concentrations in FED remained at or above baseline. As there was no significant change in ACa concentrations in either condition, this might suggest a modest effect of pre-exercise feeding on post-exercise PTH concentrations that is unrelated to calcium concentrations. Equally, however, it could reflect the effect of fasting on the PTH circadian rhythm

that reduces concentrations in the late morning compared with a normal feeding schedule (Schlemmer and Hassager, 1999).

Consistent with Studies I, II and III, the increase in PTH in the present study is not explained by a decrease in ACa concentrations. As both ACa and PTH were increased in Studies I, II and III, the increase in ACa might be explained by an increase in PTH-mediated bone resorption. The results of the present study do not support this idea, with no significant change in ACa concentrations despite a marked increase in PTH that was sustained throughout 60 min of running. Again, low lactate levels and the moderate exercise intensity suggest neither metabolic acidosis nor adrenergic agonists are likely to explain the increase in PTH.

The prompt increase in both PO_4 and PTH, and the magnitude of the increase in PO_4 (30-40%), suggest that changes in PO_4 might have stimulated the increase in PTH (Silverberg *et al.*, 1986; Kärkkäinen and Lamberg-Allardt, 1996; Martin *et al.*, 2005). However, based on the results of Study III and those of Maïmoun *et al.* (2006), which show that increases in PO_4 of a similar magnitude are not always associated with an increase in PTH, change in PO_4 seem unlikely to explain the PTH response. Again, a lack of significant correlations between changes in PO_4 and PTH during exercise in the present study supports this idea.

In conclusion, although the ingestion of a mixed meal suppressed resting β-CTX concentrations, it did not attenuate the increase in β-CTX associated with a subsequent bout of acute running. Feeding did result in a more sustained increase in β-CTX in the early recovery period although the magnitude of this effect was relatively modest. Feeding had no significant effect on the transient increase in OC and P1NP during exercise indicating that acute feeding does not favourably modulate the negative bone modelling balance associated with acute running. The lower pre-exercise and the higher post-exercise β-CTX concentrations with feeding are not explained by changes in leptin or ghrelin and were also not accompanied by changes in OPG concentrations, suggesting that OPG does not reflect acute changes in bone resorption under these conditions. Increased PTH might explain the transient increase in β-CTX during and immediately post-exercise but does not account for the different response at 2 h and 3 h post-exercise. Increased PTH did not result from decreased calcium and is unlikely to be related to the increase in PO_4.

CHAPTER VIII

THE PRO-INFLAMMATORY CYTOKINE RESPONSE TO ACUTE RUNNING

8.1 Introduction

Among the many factors known to modulate the bone turnover process, cytokines are thought to play a key role. At the time that the studies in this thesis were conceived, a number of investigations had shown that the pro-inflammatory cytokines tumour necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 can induce bone resorption both *in vitro* and *in vivo* (Thompson *et al.*, 1987; König *et al.*, 1988; Kotake *et al.*, 1996; Xing *et al.*, 2003). In addition to acting alone, these cytokines stimulate osteoclast differentiation in a synergistic fashion (Ragab *et al.*, 2003) and both stimulate RANKL production by osteoblastic cells and act synergistically with it (Kwan Tat *et al.*, 2004). Studies indicate a link between pro-inflammatory cytokines and several diseases associated with significant bone loss including post menopausal osteoporosis and rheumatoid arthritis (Pacifici, 1998; Pfeilschifter *et al.*, 2002; Siggelkow *et al.*, 2003; Wong *et al.*, 2003). Increases in these cytokines are also evident in the circulation, with serum IL-6 being predictive of femoral bone loss in postmenopausal women (Scheidt-Nave *et al.*, 2001), while a relationship between serum concentrations of pro-inflammatory cytokines and cortical bone loss in healthy older people has also been reported (Lauretani *et al.*, 2006).

An important question arising from these studies is whether the increased systemic cytokine concentrations are mediating changes in bone or if they simply reflect the extent of their expression and activity in the bone micro-environment. In this regard, the study of rheumatoid arthritis has provided some unique insights. Rheumatoid arthritis is an immune-mediated disease that is associated with chronic, local, soft tissue inflammation commonly followed by joint destruction and is initiated and maintained by interacting cascades of pro-inflammatory cytokines (Arend and Dayer, 1990). TNF-α and IL-1 are the key mediators of this process as demonstrated by the reduction in clinical and structural measures of the disease with anti-TNF-α and anti-IL-1 therapies in arthritic patients (Bresnihan *et al.*, 1998; Richard-Miceli and Dougados, 2001).

Inflammatory arthritis is also a systemic disease, with bone loss in locations distal to affected joints (Gough *et al.*, 1994; Haugeberg *et al.*, 2002; Lodder *et al.*, 2004), increased BTM concentrations (Al-Awadhi *et al.*, 1999) and increases in circulating markers and mediators of inflammation (Kutukculer *et al.*, 1998; Al-Awadhi *et al.*, 1999). The presence of increased concentrations of cytokines in the circulation in concert with systemic bone loss provides some of the strongest evidence that systemic cytokines can exert effects throughout the skeleton.

Since the late 1990's it has been known that acute, endurance exercise is associated with increases in both pro- and anti-inflammatory cytokines including TNF-α, IL-1β, IL-6 and IL-1 receptor antagonist (ra) (Ostrowski *et al.*, 1998a; Ostrowski *et al.*, 1999). These increases occur in a cascade-like manner, with modest increases in TNF-α and IL-1β and a large increase in IL-6 occurring during exercise and peaking with its termination, preceding a marked increase in IL-1ra that peaks 1-2 h later (Ostrowski *et al.*, 1998a; Ostrowski *et al.*, 1999; Ostrowski *et al.*, 2000; Nieman *et al.*, 2001). The responses of IL-6 and IL-1ra to exercise are consistent and marked, with increases of up to 100-fold being reported with prolonged, strenuous exercise (Ostrowski *et al.*, 1998a; Ostrowski *et al.*, 1998b; Ostrowski *et al.*, 1999; Toft *et al.*, 2000; Starkie *et al.*, 2001). The responses of both TNF-α and IL-1β to strenuous exercise are less marked and less consistent with some, but not all studies, observing a 1 to 2-fold increase (Nehlsen-Cannarella *et al.*, 1997; Ostrowski *et al.*, 1998a; Ostrowski *et al.*, 1998b; Ostrowski *et al.*, 1999; Moldoveanu *et al.*, 2000; Nieman *et al.*, 2001; Starkie *et al.*, 2001; Steensberg *et al.*, 2002; Starkie *et al.*, 2005). Running is of particular interest in this regard because, when examined together, studies indicate that only with running do the increases in IL-6 and IL-1ra consistently occur subsequent to increases in TNF-α and IL-1β. This suggests that, at least in part, the cytokine response to acute running might be an inflammatory response, much like the inflammatory cascade in response to trauma or infection (Dinarello, 1997).

These findings led to much speculation as to the source of the increases in cytokines during exercise. As strenuous exercising is known to induce muscle damage, one explanation for the apparent inflammatory response to running but not cycling might be a greater degree of muscle damage induced by this mode of exercise (Broussard *et al.*, 2003). However, while such a mechanism might explain increases in cytokines in concert with markers of muscle damage such as creatine kinase (CK), particularly during recovery from exercise, it does not explain the marked increases in IL-6 and IL-1ra in studies where TNF-α and IL-1β are not elevated. Subsequently, a separate line of investigations showed that a major source of the increased IL-6 in the circulation was contracting skeletal muscle (Steensberg *et al.*, 2000; Steensberg *et al.*, 2002). In contrast, there is no evidence of the release of TNF-α from muscle during exercise (Steensberg *et al.*, 2000; Steensberg *et al.*, 2002). At the time the studies in this thesis were designed, the exact biological role of the muscle-derived IL-6 was unknown, but some studies suggested it was acting in a hormone-like manner to mobilise extracellular substrates and/or augment substrate delivery during exercise (Febbraio *et al.*, 2004). It also appeared that the increase in IL-1ra with exercise could be a direct result of the increase in IL-6 (Steensberg *et al.*, 2003).

Taken together, these studies suggested two possible mechanisms by which cytokines were stimulated by acute exercise, resulting in marked increases during, and immediately after exercise, and more modest but sustained increases during recovery. Endurance runners perform daily bouts of strenuous running and are reported to display extensive evidence of muscle damage included increased CK

concentrations (Noakes, 1991; Fry, 1998; Seene *et al.*, 1999). Thus, it is feasible that this group might be exposed to a prolonged increase in cytokine concentrations through both 'metabolic' and inflammatory processes. As some endurance runners are reported to have reduced spinal BMD and, as a group, are prone to SFx injury, it is possible that a prolonged increase in cytokines resulting from strenuous training might contribute to these deleterious effects. Additionally, unaccustomed exercise is a potent inducer of muscle damage and raised levels of CK have been observed in the first month of UK Army training (Greeves, 2002). Thus, a prolonged increase in cytokines in response to the unaccustomed and strenuous nature of military training might also partly underlie the high incidence of SFx in this population. Interestingly, one study reports a significant correlation between IL-6 genotype and the percentage change in proximal femoral cross-sectional area in response to 10 weeks of military training (Dhamrait *et al.*, 2003), suggesting the involvement of IL-6 in the regulation of bone mass and bone remodelling in response to strenuous exercise.

Given the ability of cytokines to modulate the bone turnover process, possibly via the circulation, it might also be possible that changes in bone turnover reported with acute, endurance exercise, in part, result from exercise-induced increases in cytokines. At the time the studies in this thesis were conceived, this had not previously been explored and no studies had simultaneously measured cytokines and BTM in response to acute running.

The aim of this chapter was, therefore, to describe the changes in circulating concentrations of TNF-α, IL-1β, IL-6 and IL-1ra that occurred with exercise in Studies I to IV and discuss them in relation to the changes in bone turnover markers reported in Chapters IV to VII.

8.2 Materials and Methods

The methods for Study I, II, III and IV are described in Chapter 3 and Sections 4.2, 5.2, 6.2 and 7.2.

8.2.1 Treatment and storage of blood samples

For the measurement of TNF-α, IL-1β, IL-6 and IL-1ra, blood was transferred into pre-cooled tubes containing 15%, 0.12 ml of K_3E EDTA (Becton Dickinson Vacutainer System, USA) generating plasma. Tubes were gently inverted 8 to 10 times and centrifuged immediately at 2000 rpm and 5°C for 10 min. Plasma was separated and stored at -70°C until analysis.

For measurement of CK, blood was transferred into pre-cooled standard tubes (Becton Dickinson Vacutainer System, USA) and left to clot at room temperature for 60 min generating serum. Tubes were subsequently centrifuged at 2000 rpm and 5°C for 10 min and serum was separated and stored at -70°C until analysis.

8.2.2 Biochemical Analysis

Biochemical analyses were conducted as described in Table 8.1

Table 8.1 Details of biochemical analysis methods

Measure	Method
TNF-α	TNF-α, was measured using a commercial solid phase ELISA (Quantikine HS, R&D Systems Ltd, Abingdon, UK). The assay has a detection limit of 0.12 $pg \cdot mL^{-1}$ and an inter/intra assay CV of <14% across the range 0.5-32.0 $pg \cdot mL^{-1}$.
IL-1β	IL-1β was measured using ELISA (Quantikine HS, R&D Systems Ltd, Abingdon, UK). The assay has a detection limit of 0.1 $pg \cdot mL^{-1}$ and an inter/intra assay CV of <12% across the range 0.5-8.0 $pg \cdot mL^{-1}$.
IL-6	IL-6 was measured using ELISA (Quantikine HS, R&D Systems Ltd, Abingdon UK). The assay has a detection limit of 0.039 $pg \cdot mL^{-1}$ and an inter/intra assay CV of <10% across the range 0.15-10 $pg \cdot mL^{-1}$.
IL-1ra	IL-1ra was measured using ELISA (Quantikine, R&D Systems Ltd, Abingdon UK). The assay has a detection limit of 22 $pg \cdot mL^{-1}$ and an inter/intra assay CV of <8% across the range 50-3000 $pg \cdot mL^{-1}$.
CK	CK was measured using standard reagents and methodology on a P module (Roche, Lewes UK). The inter and intra assay CV across the measuring range (10-1000 $U \cdot L^{-1}$) is <5%.

8.2.3 Statistical Analysis

The general statistical analysis approach was performed as described in Section 3.3.5.

Study I: Only data for the RA and ET groups is reported. Independent samples t-tests were used to compare baseline cytokine and CK concentrations. Only raw TNF-α data met the assumptions of the LMM (normality and homogeneity). Assumptions were met for IL-1β, IL-6 and CK with a log transformation while IL-1ra required a log-square root transformation.

Study II: Despite neither reporting or displaying any symptoms of illness with fever or malaise, in SHORT, one subject had baseline concentrations of IL-6 and IL-1ra of 11.5 $ng \cdot L^{-1}$ and 1387 $ng \cdot L^{-1}$ (see Appendix C for raw data). Four additional subjects displayed normal baseline concentrations of IL-6 in both conditions but subsequently showed large and sporadic increases and decreases in IL-6 that were apparently not related to the exercise protocols (see Appendix C for raw data). However, these subjects displayed normal IL-1ra concentrations throughout both conditions. In terms of their IL-6 data, these five subjects were considered to be outliers. Due to the small ($n = 5$) group of remaining subjects, no statistical analysis was performed on IL-6 data but data from the 5 subjects is displayed graphically. Only the subject with the high IL-1ra baseline concentration was considered an outlier and statistical analysis of IL-1ra data was performed on the remaining 9 subjects. Paired samples t-tests were used to compare baseline TNF-α, IL-1β, IL-1ra and CK concentrations between the LONG and SHORT conditions. A one-way ANOVA was used to compare the change in TNF-α and IL-1β from pre- to immediately post-exercise in the four exercise bouts. As peak IL-1ra concentrations are known to occur at approximately 2 h post-exercise (Ostrowski *et al.*, 1998), the one-way ANOVA was used to compare the change in IL-1ra concentrations from pre-exercise to 2 h post-exercise. The assumptions of the LMM were achieved for TNF-α, IL-1β, IL-1ra and CK with log transformations.

Study III: IL-1β was undetectable in six subjects. One further subject was considered to be an outlier as his baseline IL-1β concentrations (3.41-4.56 $ng \cdot L^{-1}$) were 7-10 times higher than the mean of the remaining three subjects. Due to the small ($n = 3$) group of subjects remaining, no statistical analysis was performed on IL-1β data but data from the 3 subjects is displayed graphically. For the remaining cytokines, a one-way ANOVA was used to compare baseline concentrations of TNF-α, IL-6, IL-1ra and CK in the LOW, MOD and HIGH groups. The assumptions of the LMM were achieved for all cytokines with log transformations while CK required a log-square root transformation.

Study IV: Paired samples t-tests were used to compare baseline cytokine and CK concentrations. Only raw TNF-α data met the assumptions of the LMM. Assumptions were met for IL-1β, IL-6, IL-1ra and CK data with log transformations.

8.3 Results

8.3.1 Study 1

8.3.1.1 *Baseline biochemistry*

There were no significant differences in baseline concentrations of cytokines or CK between the RA and ET groups (Table 8.2).

Table 8.2. Baseline biochemistry in the recreationally-active (RA) and endurance-trained (ET) groups.

Variable	RA	ET	P Value (independent samples t-test)
TNF-α ($ng \cdot L^{-1}$)	1.39 ± 0.76	1.46 ± 1.12	P = 0.866
IL-1β ($ng \cdot L^{-1}$)	0.52 ± 0.35	0.50 ± 0.18	P = 0.897
IL-6 ($ng \cdot L^{-1}$)	0.50 ± 0.39	0.58 ± 0.47	P = 0.665
IL-1ra ($ng \cdot L^{-1}$)	239 ± 88	189 ± 54	P = 0.142
CK ($U \cdot L^{-1}$)	119 ± 66	135 ± 114	P = 0.696

Values are mean ± 1SD

8.3.1.2 *Responses to acute running*

TNF-α: There was a significant main effect of *Time* ($P < 0.001$) but no significant *Group* x *Time* interaction ($P = 0.168$) for TNF-α concentrations. Pooled, mean concentrations were significantly increased from BASE during FD ($P < 0.01$) and at EE ($P < 0.001$) (Figure 8.1, Panel A). TNF-α concentrations remained significantly ($P < 0.01$) increased during the first 2 h of recovery but were no longer different from BASE at FU1 and remained so thereafter.

IL-1β: There was a significant main effect of *Time* ($P < 0.01$) but no significant *Group* x *Time* interaction ($P = 0.118$) for IL-1β concentrations. Pooled, mean concentrations were significantly increased from BASE during FD ($P < 0.01$) and at EE ($P < 0.001$) (Figure 8.1, Panel B). IL-1β concentrations were not significantly different from BASE at 30 min post-exercise or at any time point thereafter.

IL-6: There was a significant main effect of *Time* ($P < 0.001$) but no significant *Group* x *Time* interaction ($P = 0.708$) for IL-6 concentrations. Pooled, mean concentrations were significantly ($P < 0.001$) increased from baseline during FD, at EE and during 2 h of recovery (Figure 8.1, Panel C). Peak IL-6 concentrations occurred at EE in RA (29.9 ± 13.2 $pg \cdot mL^{-1}$) and ET (31.9 ± 21.5 $pg \cdot mL^{-1}$), where they were increased 84-fold and 71-fold from BASE. During the first 2 h of recovery, concentrations remained approximately 30-fold higher than at BASE in both groups. IL-6 concentrations remained significantly ($P < 0.01$) elevated from baseline at FU1 but not thereafter.

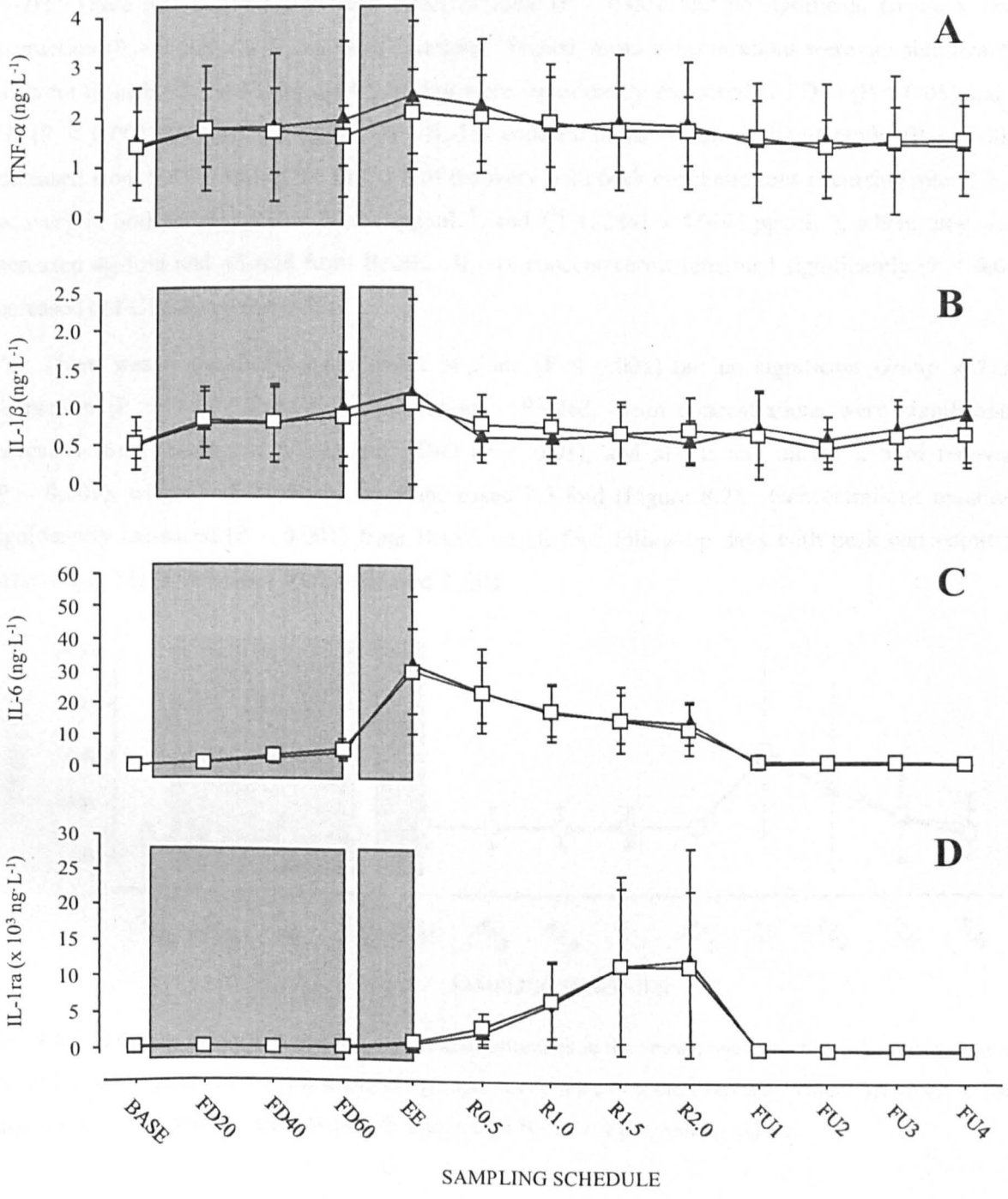

Figure 8.1. Effect of exhaustive exercise on TNF-α (Panel A), IL-1β (Panel B), IL-6 (Panel C) and IL-1ra (Panel D) concentrations in the recreationally-active (RA – open squares) and endurance-trained (ET – filled triangles) groups. Grey boxes denote exercise. Values are mean ± 1SD. Pooled, TNF-α concentrations were significantly higher than BASE throughout exercise and up to R2.0. Pooled, IL-1β concentrations were significantly higher than BASE throughout exercise only. Pooled IL-6 concentrations were significantly higher than BASE throughout exercise and up to FU1. Pooled IL-1ra concentrations were significantly higher than BASE at FD60 and up to FU1.

IL-1ra: There was a significant main effect of *Time* ($P < 0.001$) but no significant *Group* x *Time* interaction ($P = 0.468$) for IL-1ra concentrations. Pooled, mean concentrations were not significantly different from BASE at FD40 and FD60, but were significantly increased at FD60 ($P < 0.05$) and at EE ($P < 0.001$) (Figure 8.1, Panel D). IL-1ra concentrations remained significantly ($P < 0.001$) increased from BASE during the first 2 h of recovery with peak concentrations occurring after 2 h of recovery in both RA (11,874 ± 10,492 $pg \cdot mL^{-1}$) and ET (12861 ± 15492 $pg \cdot mL^{-1}$), where they were increased 40-fold and 45-fold from BASE. IL-1ra concentrations remained significantly ($P < 0.01$) increased at FU1 but not thereafter.

CK: There was a significant main effect of *Time* ($P < 0.001$) but no significant *Group* x *Time* interaction ($P = 0.966$) for CK concentrations. Pooled, mean concentrations were significantly increased from baseline at FD40 and FD60 ($P < 0.01$), and at EE and during 2 h of recovery ($P < 0.001$), where concentrations were increased 2.3-fold (Figure 8.2). Concentrations remained significantly increased ($P < 0.001$) from BASE on all four follow-up days with peak concentration occurring at FU1 where they were increased 7-fold.

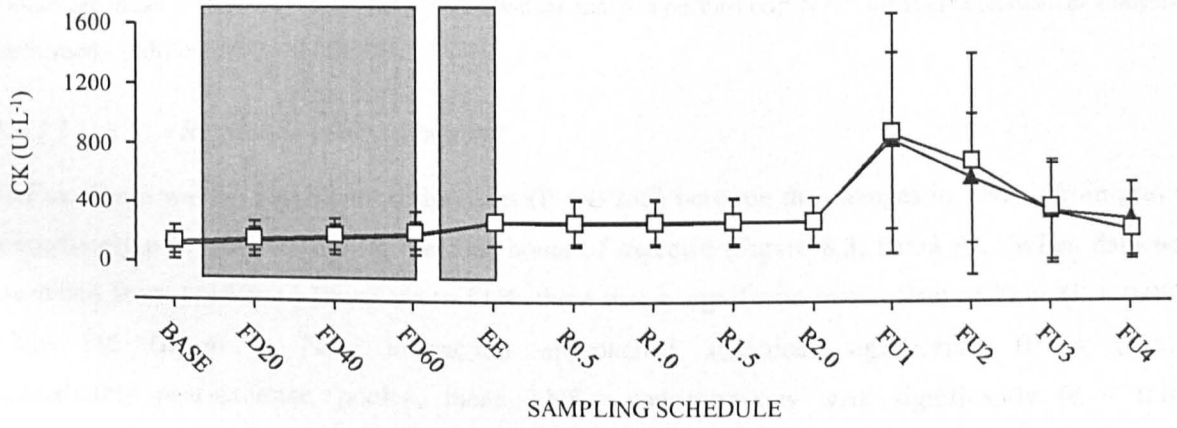

Figure 8.2. Effect of exhaustive exercise on CK concentrations in the recreationally-active (RA – open squares) and endurance-trained (ET – filled triangles) groups. Grey boxes denote exercise. Values are mean ± 1SD. Pooled, CK concentrations were significantly higher than BASE at FD40 and up to FU1.

8.3.2 Study II

8.3.2.1 Baseline biochemistry

There were no significant differences in baseline concentrations of TNF-α, IL-1β or CK between the LONG and SHORT groups although IL-1ra concentrations were 22% lower in SHORT compared with LONG (166 ± 45 *vs* 212 ± 57 ng·L^{-1}, P < 0.05) (Table 8.3).

Table 8.3. Baseline biochemistry in the LONG and SHORT groups

Variable	LONG	SHORT	P Value (paired samples t-test)
TNF-α (ng·L^{-1})	1.15 ± 0.34	1.08 ± 0.37	P = 0.461
IL-1β (ng·L^{-1})	0.46 ± 0.43	0.40 ± 0.19	P = 0.627
IL-6 (ng·L^{-1})	0.85 ± 0.60	0.1.03 ± 1.09	-
IL-1ra (ng·L^{-1})	212 ± 57	166 ± 45 [a]	P = 0.032
CK (U·L^{-1})	87 ± 19	97 ± 44	P = 0.497

Values are mean ± 1SD. N = 5 for IL-6 (no statistical analysis performed); N = 9 for IL-1ra (statistical analysis performed). [a] different (P < 0.05) from LONG.

8.3.2.2 Responses to acute running

TNF-α: There was no significant differences (P = 0.255) between the changes in TNF-α from pre- to immediately post-exercise during the four bouts of exercise (Figure 8.3, Panel A). When data was examined from 1415 h on Day 5 up to FU4, there was a significant main effect of *Time* (P < 0.001) while the *Group* x *Time* interaction approached statistical significance (P = 0.052). Immediately post-exercise, pooled, mean TNF-α concentrations were significantly (P < 0.05) increased from pre-exercise by 46%, although this increase appeared mostly due to an increase in the SHORT condition (Figure 8.4, Panel A). Concentrations had returned to pre-exercise levels by 1 h post-exercise and there were no further significant differences thereafter.

IL-1β: There was no significant differences (P = 0.966) between the changes in IL-1β from pre- to immediately post-exercise during the four bouts of exercise (Figure 8.3, Panel B). When data was examined from 1415 h on Day 5 up to FU4, there was a significant main effect of *Time* (P < 0.001) but no significant *Group* x *Time* interaction (P = 0.527). Immediately post-exercise, pooled, mean IL-1β concentrations were significantly (P < 0.01) increased from pre-exercise by 2.5-fold (Figure 8.4, Panel B). Concentrations had returned to pre-exercise levels by 1 h post-exercise and there were no further significant differences thereafter.

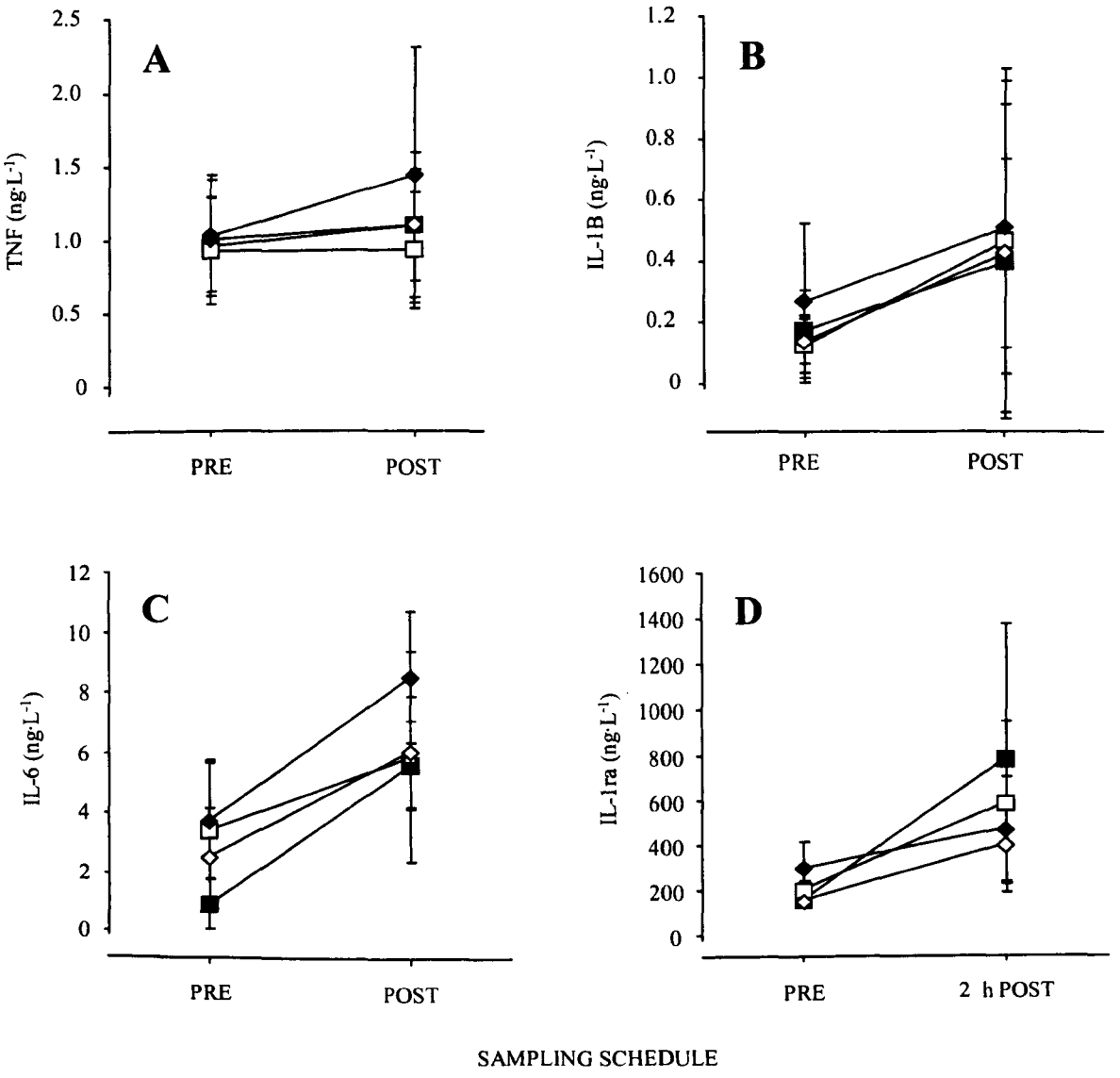

Figure 8.3. Change in TNF-α (Panel A), IL-1β (Panel B), IL-6 (Panel C) and IL-1ra (Panel D) with L-ExA (filled squares), L-ExB (open squares), S-ExA (open diamonds), S-ExB (filled diamonds). Values are mean ± 1SD. Change in concentrations for TNF-α, IL-1β and IL-6 are measured from pre- to immediately post-exercise and from pre-exercise to 2 h post-exercise for IL-1ra. N = 5 for IL-6 (no statistical analysis performed). N = 9 for IL-1ra (statistical analysis performed).

IL-6: The change in IL-6 concentrations from pre- to immediately post-exercise during the four exercise bouts in 5 subjects is displayed Figure 8.3, Panel C. Changes in IL-6 concentrations from 1415 h on Day 5 up to FU4 in 5 subjects are displayed in Figure 8.4, Panel C.

IL-1ra: There was a tendency for different changes in IL-1ra from pre- to immediately post-exercise during the four exercise bouts but this did not reach the assigned level of statistical significance (P = 0.073) (Figure 8.3, Panel D).

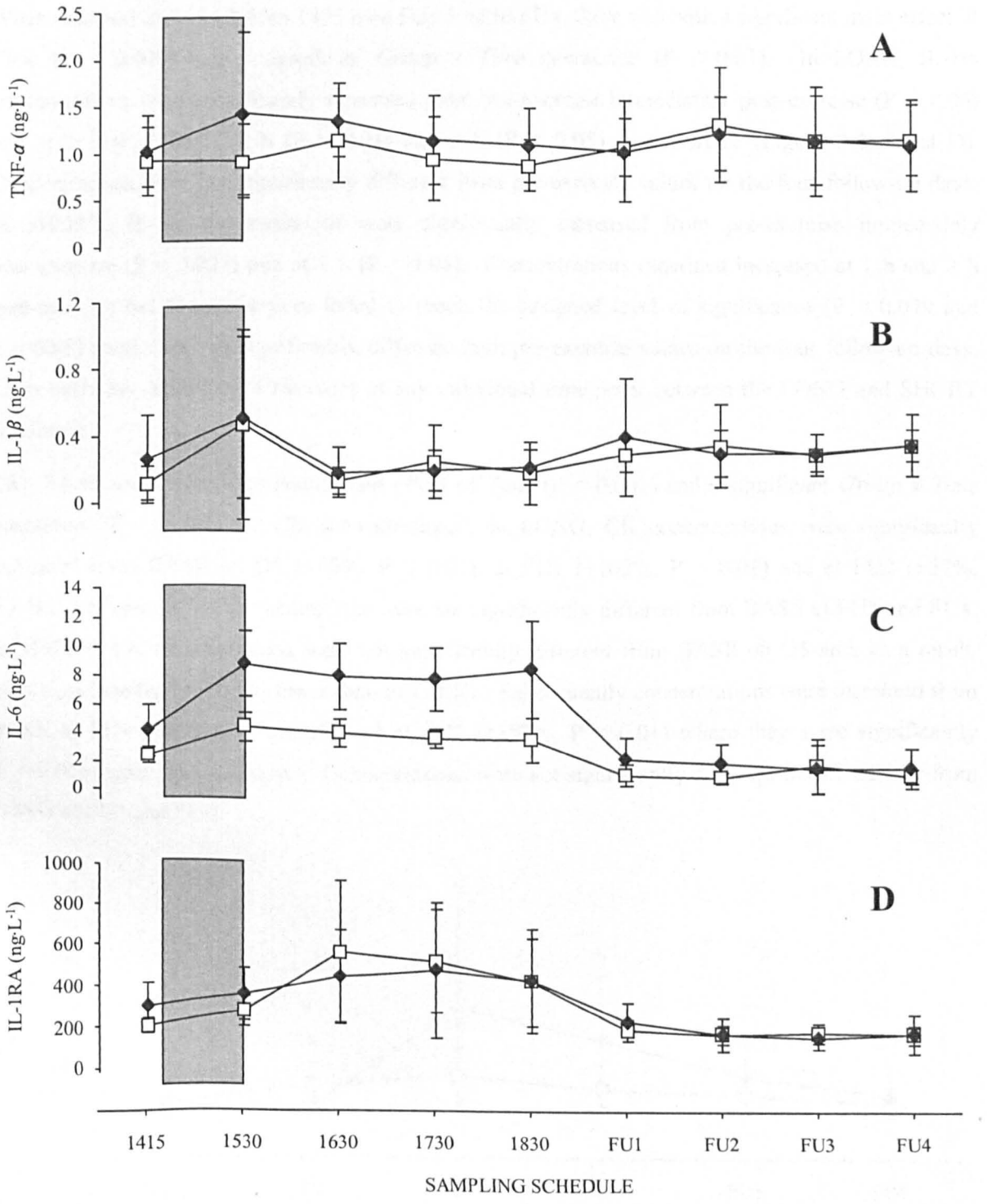

Figure 8.4. TNF-α (Panel A), IL-1β (Panel B), IL-6 (Panel C) and IL-1ra (Panel D) concentrations before (1415) and after (1530 h – 1830 h) exercise bout B (ExB) on Day 5, and on four follow-up days (FU1 – FU4) in the LONG (open squares) and SHORT (filled diamonds) conditions. Grey box denotes ExB. Values are mean ± 1SD. N = 5 for IL-6 (no statistical analysis performed). N = 9 for IL-1ra (statistical analysis performed). Pooled, TNF-α concentrations were significantly higher than 1415 h at 1530 h only. Pooled, IL-1β concentrations were significantly higher than 1415 h at 1530 h only. Pooled IL-1ra concentrations were increased from 1415 h at 1630 h and up to 1830 h.

When data was examined from 1415 h on Day 5 up to FU4, there was both a significant main effect of *Time* ($P < 0.001$) and a significant *Group* x *Time* interaction ($P < 0.01$). In LONG, IL-1ra concentrations were significantly increased from pre-exercise immediately post-exercise ($P < 0.05$) and at 1 h ($P < 0.05$), 2 h ($P < 0.01$) and 3 h ($P < 0.05$) post-exercise (Figure 8.4, Panel D). Concentrations were not significantly different from pre-exercise values on the four follow-up days. In SHORT, IL-1ra concentrations were significantly increased from pre-exercise immediately post-exercise ($P < 0.001$) and at 1 h ($P < 0.05$). Concentrations remained increased at 1 h and 2 h post-exercise but the differences failed to reach the assigned level of significance ($P = 0.079$ and $P = 0.087$) and were not significantly different from pre-exercise values on the four follow-up days. There were no significant differences at any individual time point between the LONG and SHORT conditions.

CK: There was both a significant main effect of *Time* ($P < 0.001$) and a significant *Group* x *Time* interaction ($P < 0.001$) for CK concentrations. In LONG, CK concentrations were significantly increased from BASE on D5 (+65%, $P < 0.05$), at FU1 (+102%, $P < 0.01$) and at FU2 (+57%, $P < 0.01$) (Figure 8.5). Concentrations were not significantly different from BASE at FU3 and FU4. In SHORT, CK concentrations were not significantly different from BASE on D5 and, as a result, were significantly ($P < 0.05$) lower than in LONG. Subsequently concentrations were increased from BASE at FU1 (+286%, $P < 0.05$) and at FU2 (+187%, $P < 0.01$) where they were significantly ($P < 0.05$) higher than in LONG. Concentrations were not significantly different from BASE or from LONG at FU3 and FU4.

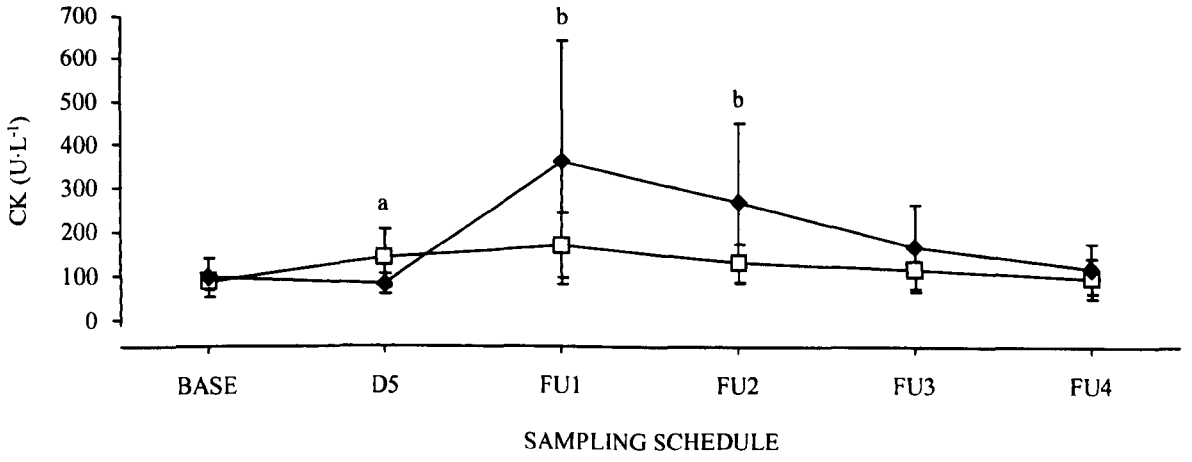

Figure 8.5. CK concentrations at baseline (BASE), at 0800 on Day 5 (Day 5), and on the four follow-up days (FU1 – FU4) in the LONG (open squares) and SHORT (filled diamonds) conditions. Values are mean ± 1SD. In LONG, CK concentrations were significantly higher than BASE at D5 and up to FU2. Concentrations in SHORT were significantly higher than BASE at FU1 and FU2 only. [a] SHORT different ($P < 0.05$) from LONG; [b] SHORT different ($P < 0.01$) from LONG.

8.3.3 Study III

8.3.3.1 Baseline biochemistry

There were no significant differences in baseline concentrations of cytokines or CK between the LOW, MOD and FAST groups (Table 8.4).

Table 8.4. Baseline biochemistry in the LOW, MOD and HIGH groups.

Variable	LOW	MOD	HIGH	P Value (one-way ANOVA)
TNF-α ($ng \cdot L^{-1}$)	1.7 ± 0.4	1.8 ± 0.4	1.9 ± 0.4	P = 0.381
IL-1β ($ng \cdot L^{-1}$)	0.41 ± 0.04	0.38 ± 0.26	0.37 ± 0.30	-
IL-6 ($ng \cdot L^{-1}$)	0.94 ± 0.59	0.73 ± 0.27	0.70 ± 0.28	P = 0.381
IL-1ra ($ng \cdot L^{-1}$)	160 ± 56	137 ± 50	133 ± 53	P = 0.488
CK ($U \cdot L^{-1}$)	100 ± 33	122 ± 75	127 ± 57	P = 0.556

Values are mean ± 1SD. N = 3 for IL-1β (no statistical analysis performed).

8.3.3.2 Responses to acute running

TNF-α: There was a significant main effect of *Time* ($P < 0.01$) but no significant *Group* x *Time* interaction ($P = 0.214$) for TNF-α concentrations. Pooled, mean concentrations were significantly ($P < 0.01$) increased from BASE at EX60 and remained increased ($P < 0.05$) at R0.5 (Figure 8.6, Panel A). Concentrations were not significantly different from BASE at R1.0 and there were no further differences thereafter.

IL-1β: Changes in IL-1β in three subjects are shown in Figure 8.6, Panel B.

IL-6: There was a both significant main effect of *Time* ($P < 0.001$) and a significant *Group* x *Time* interaction ($P = 0.001$) for IL-6. In LOW, IL-6 concentrations were significantly increased from BASE at EX40 ($P < 0.001$) and remained elevated throughout exercise ($P < 0.001$) and the first 3 h of recovery ($P < 0.001$) (Figure 8.6, Panel C). Peak concentrations occurred at R3.0 where they were increased 530%. In MOD, concentrations were also significantly ($P < 0.001$) increased from BASE from EX40 through to R3.0. Peak concentrations occurred at EX60 when they were increased 640%. There were no differences between the LOW and MOD conditions at any time point. In HIGH, concentrations were significantly increased from BASE at EX20 ($P < 0.05$) and had increased by 500% at EX40 ($P < 0.001$). Peak concentrations occurred at EX60 where they were increased ($P < 0.001$) 1200% from BASE and were significantly higher than in both LOW ($P < 0.01$) and MOD ($P < 0.05$). Concentrations remained significantly ($P < 0.001$) increased from BASE in the first 3 h of recovery but were not significantly different from LOW or MOD. IL-6 concentrations were not significantly different from BASE in any group at FU1 or thereafter.

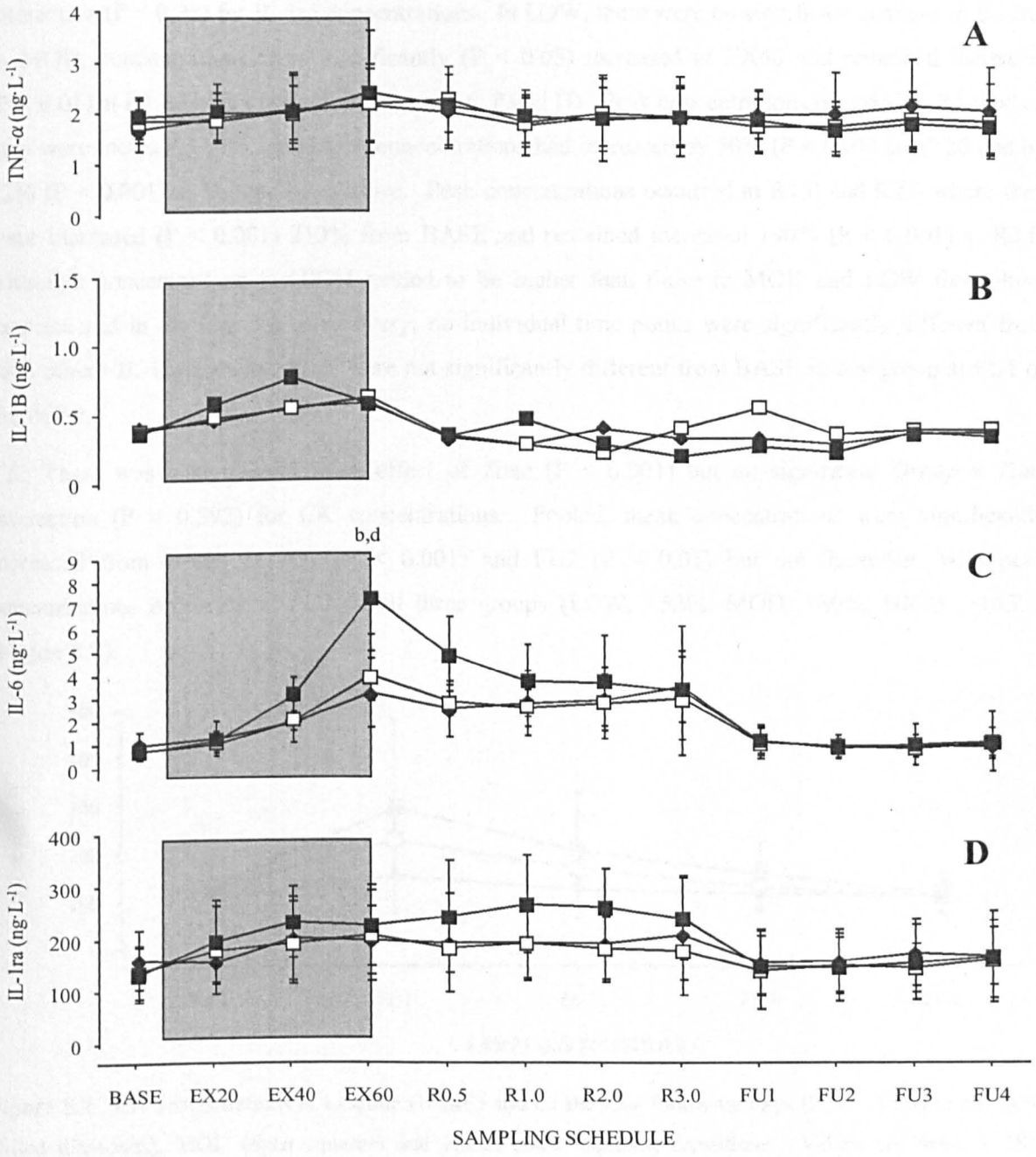

Figure 8.6. Changes in TNF-α (Panel A), IL-1β (Panel B), IL-6 (Panel C) and IL-1ra (Panel D) concentrations at baseline (BASE), during 60 min of exercise (EX20 – EX60), during 3 h of recovery (R0.5 – R3.0) and on 4 follow up days (FU1 – FU4) in the LOW (filled diamonds), MOD (open squares) and HIGH (filled squares) conditions. Values are mean ± 1SD. Grey box denotes exercise. N = 3 for IL-1β data. Pooled, TNF-α concentrations were significantly higher than BASE at EX60 and R0.5 only. In LOW and MOD, IL-6 concentrations were significantly higher than BASE at EX40 and up to R3.0. In HIGH, IL-6 concentrations were significantly higher than BASE at EX20 and up to R3.0. Pooled IL-1ra concentrations were higher than BASE at FD60 and up to FU1. There was no significant change in IL-1ra concentrations in LOW. Concentrations were significantly higher than BASE at EX40 and up to R1.0 in MOD, and from EX20 to R3.0 in HIGH. [b] HIGH different ($P < 0.01$) from LOW; [d] HIGH different ($P < 0.05$) from MOD.

IL-1ra: There was a both significant main effect of *Time* ($P < 0.001$) and a significant *Group* x *Time* interaction ($P < 0.05$) for IL-1ra concentrations. In LOW, there were no significant changes in IL-1ra. In MOD, concentrations were significantly ($P < 0.05$) increased at EX40 and remained increased ($P < 0.01$) at R1.0 but not thereafter (Figure 8.6, Panel D). Peak concentrations occurred at R1.0 when they were increased 56%. In HIGH, concentrations had increased by 50% ($P < 0.01$) at EX20 and by 72% ($P < 0.001$) at the end of exercise. Peak concentrations occurred at R1.0 and R2.0 where they were increased ($P < 0.001$) 210% from BASE and remained increased 190% ($P < 0.001$) at R3.0. Although concentrations in HIGH tended to be higher than those in MOD and LOW throughout exercise and in the first 3 h of recovery, no individual time points were significantly different from each other. IL-1ra concentrations were not significantly different from BASE in any group at FU1 or thereafter.

CK: There was a significant main effect of *Time* ($P < 0.001$) but no significant *Group* x *Time* interaction ($P = 0.592$) for CK concentrations. Pooled, mean concentrations were significantly increased from BASE at FU1 ($P < 0.001$) and FU2 ($P < 0.01$) but not thereafter, with peak concentrations occurring at FU1 in all three groups (LOW, +52%; MOD, +80%; HIGH, +163%) (Figure 8.7).

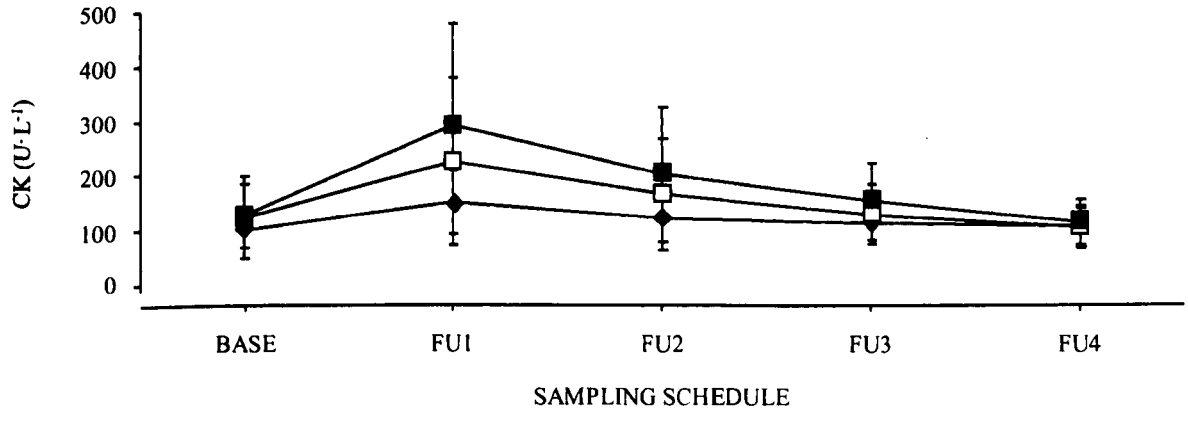

Figure 8.7. CK concentrations at baseline (BASE) and on the four follow-up days (FU1 – FU4) in the LOW (filled diamonds), MOD (open squares) and HIGH (filled squares) conditions. Values are mean ± 1SD. Pooled, CK concentrations were significantly higher than BASE at FU1 and FU2 only.

8.3.4 Study IV

8.3.4.1 Baseline biochemistry

There were no significant differences in baseline concentrations of cytokines or CK between the FAST and FED groups (Table 8.5).

Table 8.5. Baseline biochemistry in the FAST and FED groups.

Variable	FAST	FED	P Value (paired samples t-test)
TNF-α ($ng \cdot L^{-1}$)	1.70 ± 0.47	1.72 ± 0.46	P = 0.847
IL-1β ($ng \cdot L^{-1}$)	0.45 ± 0.38	0.50 ± 0.43	P = 0.489
IL-6 ($ng \cdot L^{-1}$)	0.93 ± 0.70	0.79 ± 0.36	P = 0.622
IL-1ra ($ng \cdot L^{-1}$)	183 ± 69	205 ± 62	P = 0.352
CK ($U \cdot L^{-1}$)	144 ± 78	128 ± 72	P = 0.672

Values are mean ± 1SD.

8.3.4.2 *Responses to acute running*

TNF-α: There was no significant main effect of *Time* (P = 0.439) and no significant *Group* x *Time* interaction (P = 0.946) for TNF-α concentrations (Figure 8.8, Panel A).

IL-1β: There was no significant main effect of *Time* (P = 0.518) and no significant *Group* x *Time* interaction (P = 0.742) for IL-1β concentrations (Figure 8.8, Panel B).

IL-6: There was a significant main effect of *Time* ($P < 0.001$) but no significant *Group* x *Time* interaction (P = 0.997) for IL-6 concentrations. Pooled, mean concentrations were not significantly different from BASE prior to exercise but were significantly ($P < 0.01$) increased at EX60, where mean concentrations were increased 6.5 to 7.5-fold (Figure 8.8, Panel C). Concentrations declined in the first 3 h post-exercise but remained significantly increased ($P < 0.001$) from BASE at R3.0 with concentrations increased 4.5 to 5.5-fold. Concentrations were not significantly different from BASE on any of the follow-up days.

IL-1ra: There was a significant main effect of *Time* ($P < 0.001$) but no significant *Group* x *Time* interaction (P = 0.698) for IL-1ra concentrations. Pooled, mean concentrations were not significantly different from BASE prior to exercise but were significantly increased from BASE at EX30 ($P < 0.001$) and at EX60 ($P < 0.001$) (Figure 8.8, Panel D). Concentrations remained significantly increased from BASE at R1.0 ($P < 0.001$), R2.0 ($P < 0.001$) and R3.0 ($P < 0.01$) with peak concentrations occurring at EX60 and R2.0 in FAST and FED, increased 74% and 90% from BASE.

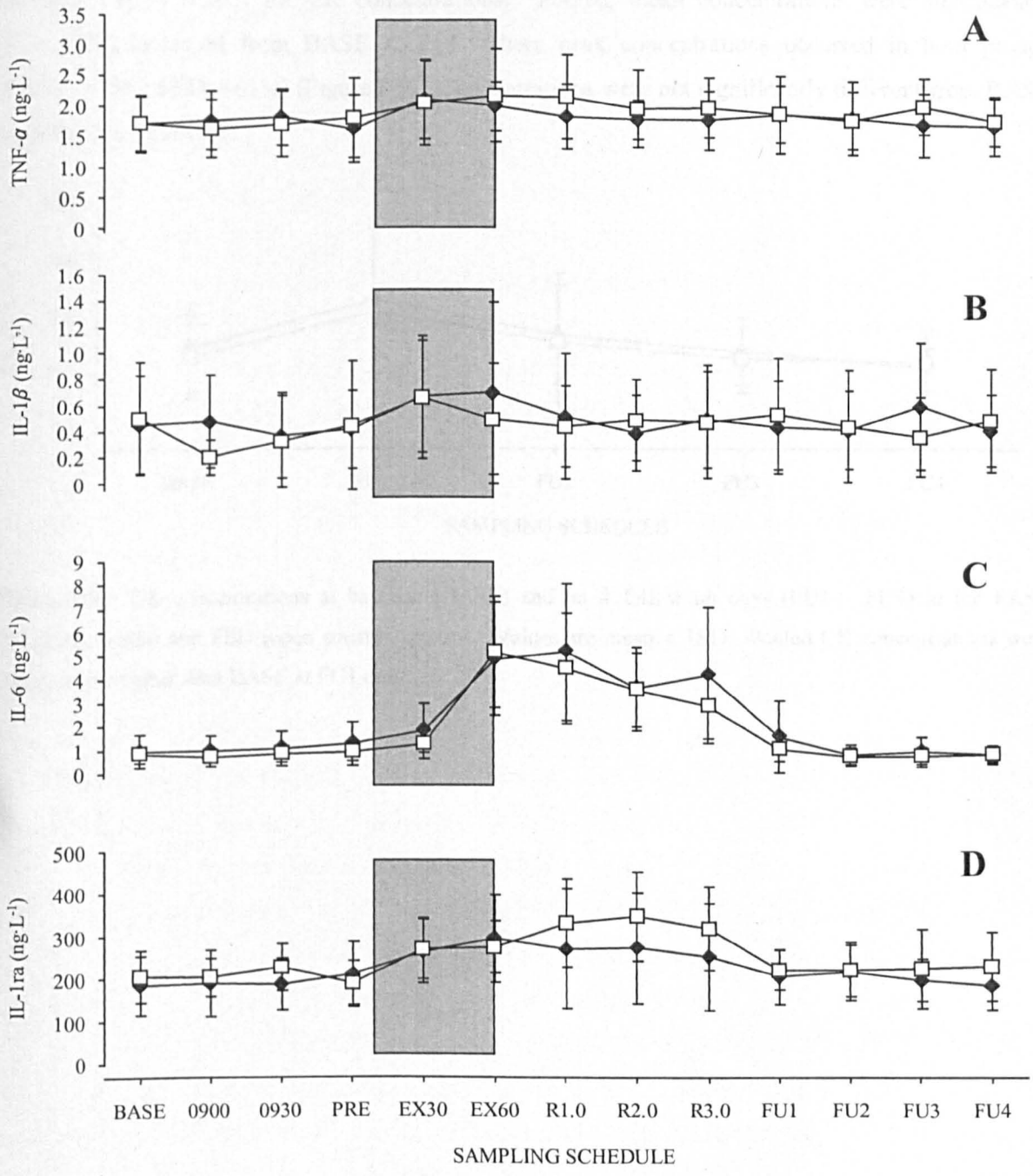

Figure 8.8. Changes in TNF-α (Panel A), IL-1β (Panel B), IL-6 (Panel C) and IL-1ra (Panel D) concentrations at baseline (BASE), before (0900 - PRE), during (EX30) and for 3 h after exercise (EX60 - R3.0), and on 4 follow up days (FU1 – FU4) in the FAST (filled diamonds) and FED (open squares) groups. Grey box denotes exercise. Values are mean ± 1SD. Pooled IL-6 concentrations were significantly higher than BASE at EX30 and up to R3.0. Pooled IL-1ra concentrations were significantly higher than BASE at EX30 and up to R3.0.

CK: There was a significant main effect of *Time* ($P < 0.001$) but no significant *Group* x *Time* interaction ($P = 0.503$) for CK concentrations. Pooled, mean concentrations were significantly ($P < 0.01$) increased from BASE at FU1, where peak concentrations occurred in both groups (FAST, +75%; FED, +61%) (Figure 8.9). Concentrations were not significantly different from BASE from FU2 or thereafter.

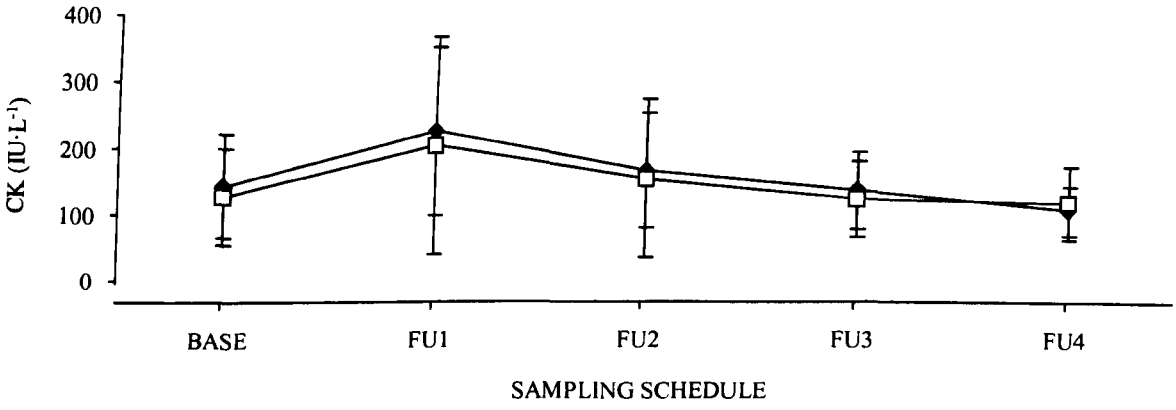

Figure 8.9. CK concentrations at baseline (BASE) and on 4 follow up days (FU1 – FU4) in the FAST (filled diamonds) and FED (open squares) groups. Values are mean ± 1SD. Pooled CK concentrations were significantly higher than BASE at FU1 only.

8.3 Discussion

The main findings from this chapter are that 1) acute running was generally associated with a modest and transient increase in TNF-α that was most prolonged (up to 2 h post-exercise) with exhaustive exercise and tended to be larger with reduced recovery; 2) increases in IL-1β with exercise are modest and transient, even with exhaustive exercise; 3) a marked increase in IL-6 occurred during acute running, the magnitude of which was greatest with exhaustive exercise, and was increased with increasing exercise intensity and possibly also with reduced recovery; 4) all bouts of exercise were followed by a marked increase in IL-1ra that generally peaked at 1-2 h post-exercise, was most marked following exhaustive exercise, and tended to be larger with reduced recovery duration and; 5) despite marked increases in CK following all exercise protocols, particularly exhaustive exercise and reduced recovery, there was little evidence of increased cytokine concentrations on the four follow-up days.

The findings from this chapter confirm those from previous investigations of running, which report a cascade-link increase in cytokines, with modest increases in TNF-α and IL-1β and a marked increase in IL-6, preceding a marked increase in IL-1ra (Ostrowski *et al.*, 1998a; Ostrowski *et al.*, 1999; Ostrowski *et al.*, 2000; Nieman *et al.*, 2001). The categorisation of the exercise-associated cytokine changes as an 'inflammatory' response relies on increases in TNF-α and IL-1β preceding those in IL-6 and IL-1ra. As the majority of previous studies of acute running report increased TNF-α either in concert or preceding changes in IL-6 and IL-1ra, it is possible that at least part of the cytokine response to running is, indeed, inflammatory. Interestingly, all of these studies have examined long duration, highly strenuous running and not all studies of acute running report increases in TNF-α (Drenth *et al.*, 1995; Suzuki *et al.*, 2003). Although TNF-α was increased in Studies I-III, 60 min of running at 65% VO_{2max} in Study IV was associated with increases in IL-6 and IL-1ra despite no change in TNF-α or IL-1β. This suggests that at least some of the increase in IL-6 with running does not represent part of an inflammatory cascade. Additionally, to what extent the increase in TNF-α itself is related to inflammation is also unclear, with reports of either no change (Starkie *et al.*, 2005), or a reduced number (Starkie *et al.*, 2001) and percentage (Starkie *et al.*, 2000) of monocytes spontaneously producing TNF-α with exercise.

Observations of large increases in IL-6 concentrations without increases in TNF-α or IL-1β, prompted a series of investigations into the possible functions and factors regulating its appearance in the circulation and these studies showed that skeletal muscle was a major source of IL-6 during exercise (Steensberg *et al.*, 2000; Steensberg *et al.*, 2002). As its appearance in the circulation is most marked with long duration, strenuous exercise, is attenuated by carbohydrate ingestion (Nehlsen-Cannarella *et al.*, 1997), and increased by low muscle glycogen (Keller *et al.*, 2001; Steensberg *et al.*, 2001) and exercise intensity (Helge *et al.*, 2003), it has been suggesting that one function of IL-6 during exercise is to mediate endogenous glucose production (Febbraio *et al.*, 2004). Similarly, in the present studies,

the most marked increase in IL-6 occurred with the longest and most strenuous bout of exercise (Study I) and the increase was greater with increased exercise intensity (Study III). Also, although only based on 5 subjects, the results of Study II suggest the increase in IL-6 with exercise is enhanced with a short recovery period between two bouts of exercise. This would corroborate previous findings from a study in cycling (Ronsen *et al.*, 2002) and be consistent with the influence of pre-exercise muscle glycogen levels on IL-6 concentrations (Keller *et al.*, 2001; Steensberg *et al.*, 2001). Taken together, these studies suggest that the increased IL-6 concentrations in the present studies might also be explained by increased release from skeletal muscle, possibly functioning in an endocrine manner in the regulation of endogenous glucose production (Febbraio *et al.*, 2004).

The purpose of this chapter was to explore a novel hypothesis that increases in circulating cytokines during acute running might, in part, be mediating changes in bone turnover. Such a mechanism might function in two ways: through marked increases in cytokines during exercise and/or with a more modest but more prolonged increase in basal concentrations in the hours and days following exercise. The following section will discuss this hypothesis in light of the evidence presented in this chapter and Chapters IV to VII, and with regard to evidence published since the studies in this thesis were conceived and conducted.

Regarding the acute cytokine response, changes in circulating IL-1β in all studies were modest and transient, even with exhaustive exercise in Study I, where concentrations had returned to baseline by 30 min post-exercise. Although transient, exercise in Study I lasted more than 2 h for most subjects, making this the most sustained increase in IL-1β across the four studies. As Study I was the only study to show a sustained increase in β-CTX on the follow-up days, this might suggest the involvement of changes in circulating IL-1β. However, importantly, in Study IV there was a marked but more transient increase in β-CTX despite no change in IL-1β concentrations suggesting no involvement of systemic IL-1β concentrations.

As with IL-1β, increases in TNF-α were both modest and transient with the most prolonged increase occurring in Study I with concentrations increased for 2 h post-exercise. Again, this could indicate a role of circulating TNF-α in increased bone resorption with running but, as with IL-1β, in Study IV there was no significant change in TNF-α when β-CTX was transiently increased. Additionally, as shown in these studies, acute running is also associated with a marked increase with IL-1ra as well as soluble TNF receptors (Tilz *et al.*, 1993; Ostrowski *et al.*, 1999) during, and in the hours immediately following exercise. It is possible, therefore, that any pro-inflammatory effects of small and transient increases in circulating IL-1β or TNF-α are balanced by increases in these anti-inflammatory molecules.

By far the largest increases with exercise occurred in IL-6 concentrations. In Study I, where a sustained increase in β-CTX was observed, mean IL-6 concentrations were increased 71- and 84-fold in RA and ET, at least 10 times the magnitude of increases seen in the other studies. Additionally, only in Study I did IL-6 concentrations remain elevated on the follow-up days (albeit only modestly at FU1) indicating both a larger and more prolonged elevation in concentrations. It is possible, therefore, that this more pronounced increase in IL-6 contributed to the sustained increase in bone resorption evident only in Study I. In the remaining studies, however, the only evidence in support of this idea comes from Study III, where the increase in IL-6 in HIGH was greater than in MOD and LOW and subsequently β-CTX concentrations tended to be higher in HIGH in the first hour post-exercise. In contrast, in the SHORT condition in Study II, IL-6 concentrations were continually increased during the course of Day 5, especially after ExB, but β-CTX concentrations were not significantly different from baseline on any of the recovery days. Additionally, in Study IV, feeding suppressed resting β-CTX concentrations but had no effect on IL-6, while subsequent increases in IL-6 with exercise do not explain the different β-CTX responses in FAST and FED at 2 and 3 h post-exercise.

Taken together, this small group of exploratory studies provides no clear evidence that the acute cytokine response to exercise might be involved in the changes in bone resorption associated with acute running. Based on these studies, should any effect exist, the most likely mechanism might be via a marked and sustained increase in IL-6 with long-duration, strenuous running.

The second mechanism by which changes in circulating cytokines might mediate changes in bone turnover is as part of a chronic inflammatory response to exercise-induced tissue damage, resulting in a more modest but more prolonged increase in basal cytokine concentrations. Based on these studies there is little evidence to support such a mechanism as, despite marked increases in CK, a marker of muscle damage, cytokines were not significantly increased from BASE on the four follow-up days. Due to the short duration of these studies, this finding does not preclude the possibility that a local, acute inflammatory response to tissue damage could become chronic, and then systemic, and subsequently stimulate bone turnover. It is possible that only once the inflammatory response becomes chronic, such as with repeated bouts of strenuous exercise and/or insufficient recovery, will cytokines be released into the circulation, stimulating circulating monocytes to produce further cytokines (Smith, 2000). However, a delayed increase in cytokines associated with exercise-induced muscle damage appears unlikely to be involved in the marked increase in β-CTX observed in Study I.

During the time that the studies in this thesis were conceived and performed, further information has become available that has relevance in the discussion of a possible link between circulating cytokines and changes in bone turnover. Based on the studies in this thesis, compared to IL-6, changes in TNF-α and IL-1β with exercise appear less likely to mediate a change in bone turnover. However, it is rheumatoid arthritis, a disease characterised by enhanced local TNF-α and IL-1β activity and systemic

bone loss, that provides some of the most compelling evidence for a cytokine link. For example, there is now additional evidence of the role of inflammation in systemic bone loss in rheumatoid arthritis, with van Staa *et al.* (2006) reporting an independent role of disease activity and duration on the risk of osteoporotic fracture at numerous skeletal sites, including the hip. Additionally, epidemiological studies show that c-reactive protein levels predict osteoporotic fracture independent of other established risk factors (Schett *et al.*, 2006), while prolonged, systemic administration of IL-1β at supraphysiological concentrations increases bone resorption and decreases bone mineral content without any evidence of effects in joint tissue (del Pozo *et al.*, 2005). Indeed, IL-1 appears to be essential for TNF-α-mediated systemic bone loss, with mice deficient in IL-1 completely protected from inflammatory bone loss induced by TNF- α (Polzer *et al.*, 2010).

Recent animal data, however, suggests that, although inhibition of TNF-α or IL-1 reduces both local and systemic inflammation, neither prevents bone loss in distant lumbar vertebrae (Stolina *et al.*, 2009). In contrast, the suppression of RANKL using OPG prevents both local and systemic bone loss, despite having no effect on inflammatory markers at the local or systemic level. Taken together, these findings question the direct role of increased serum levels of TNF-α or IL-1 in systemic changes in bone resorption.

IL-6 levels are also increased in the joints and serum of patients with rheumatoid arthritis. IL-6 signalling acts via at least one sub-unit of the signal transduction unit gp130. Although the expression of gp130 is ubiquitously, the expression of the IL-6 receptor (IL-6R) is limited to hepatocytes, leucocytes (Rose-John, 2003) and some but not all adipocytes (Bastard *et al.*, 2002). A naturally occurring soluble receptor (sIL-6R) also exists in the circulation which allows the formation of the IL-6/IL-6R complex and increases the half-life of IL-6 (Peters *et al.*, 1996). This complex can be rendered inactive by a soluble form of the gp130 receptor (sgp130) (Jostock *et al.*, 2001). In the presence of sIL-6R, the ubiquitous expression of gp130 renders almost all cells sensitive to IL-6 (Peters *et al.*, 1998). Osteoclasts have no or very little expression of functional IL-6 in physiological conditions (Tamura *et al.*, 1993) and, while neither IL-6 nor sIL-6R induce osteoclast formation directly, the complex of IL-6 with sIL-6R is highly effective (Tamura *et al.*, 1993). Indeed, sIL-6R has been implicated in several conditions associated with bone loss including post menopausal osteoporosis (Masiukiewicz *et al.*, 2002) and rheumatoid arthritis (Desgeorges *et al.*, 1997).

Although sIL-6R is present in the serum of healthy individuals, as of 2005, there was no evidence of an increase in its concentrations with acute exercise (Neidhart *et al.*, 2000; Keller *et al.*, 2005a, 2005c). However, subsequently, several studies report increases in sIL-6R following endurance cycling (Gray *et al.*, 2008; Gray *et al.*, 2009). This type of exercise also increases the IL-6/sIL-6R complex for up to 1.5 h post-exercise (Gray *et al.*, 2009), while six consecutive days of long-duration cycling is associated with a persistent increase in sIL-6R from the second day (Robson-Ansley *et al.*,

2009). Although sgp130 is also increased during exercise (Gray *et al.*, 2008), the ELISA developed by Gray *et al.* (2009) measures only IL-6 bound to sIL-6R and not the tertiary IL-6/sIL-6R/sgp130 complex, suggesting an increase in the biologically active IL-6/IL-6R complex during exercise. Taken together, the basal expression of IL-6R by hepatocytes and adipocytes, and increases in IL-6 and sIL-6R in serum and IL-6R in muscle (Keller *et al.*, 2005a; Keller *et al.*, 2005c) with exercise, are consistent with recent data suggesting that the primary function of IL-6 during exercise is to increase glucose uptake and fat oxidation in muscle and, in an endocrine manner, increase hepatic glucose production or lipolysis in adipose tissue (Pedersen and Febbraio, 2008).

In addition to these proposed functions, given the need for both IL-6 and sIL-6R for osteoclast formation, and the increased serum levels of both IL-6 and sIL-6R in arthritis patients, it is also possible that, in combination with increased levels of sIL-6R, the increase in serum IL-6 with exercise might be capable of stimulating bone resorption. As an infusion of PTH increases circulating concentrations of IL-6, sIL-6R and bone resorption markers (Masiukiewicz *et al.*, 2002), it is possible that increased PTH concentrations during exercise might mediate such a mechanism. It is well known that PTH induces IL-6 expression by osteoblasts (Feyen *et al.*, 1989; Ishimi *et al.*, 1990) but Mitnick *et al.* (2001) also report the release of both IL-6 and sIL-6R from the liver during PTH infusion. This finding suggests that the increase in bone resorption with PTH infusion might, in part, also be stimulated systemically by increased IL6/sIL-6R. During 120 min of cycling at 65% VO_{2max}, however, the liver does not contribute to the systemic increase in IL-6 and, in fact, participates in its removal (Febbraio *et al.*, 2003). Although the study of Febbraio *et al.* (2003) did not measure PTH or sIL-6R, 2 h of moderate intensity cycling is reported to increase PTH concentrations (Barry and Kohrt, 2007). Therefore, it remains possible that the increase in sIL-6R during exercise (Gray *et al.*, 2008; Gray *et al.*, 2009) results from a PTH-mediated hepatic release. Thus, increased PTH leading to increased sIL-6R might provide a mechanism by which increased systemic IL-6 could exert an effect on bone resorption.

Much of this argument rests on a fuller understanding of the targets and actions of IL-6, which are known to be wide ranging, and its signalling pathways complex. This is further highlighted by recent data that indicates that, both upstream and downstream, signalling pathways for IL-6 differ markedly between myocytes and macrophages, with signalling in monocytes/macrophages creating a pro-inflammatory response, whereas in muscle, IL-6 activation is totally independent of a preceding TNF-response (Brandt and Pederson, 2010). Additionally, despite IL-6R blockade directly inhibiting osteoclast formation in arthritic mice, it does so independently of changes in local inflammation and is unable to prevent TNF-mediated systemic bone loss (Axmann *et al.*, 2009). This finding raises further questions regarding the role of circulating IL-6 in modulating bone resorption throughout the skeleton.

In conclusion, this chapter has shown that acute running is associated with modest and inconsistent effects on TNF-α and IL-1β concentrations but marked increases in IL-6, the latter of which is greatest with exhaustive exercise, is increased with increasing exercise intensity, and also possibly with reduced recovery between two bouts of exercise. All bouts of running were followed by a marked increase in IL-1ra, an anti-inflammatory cytokine that peaks in the first few hours post-exercise. When examined in relation to the results of Chapters IV-VII, from this limited data, there is no clear evidence that the acute cytokine response to exercise is involved in the changes in bone resorption observed. However, based on the findings from Study I, as well as those from several recently published studies, an effect of a substantial increase in circulating IL-6 in conjunction with an increase in sIL-6R remains a possibility. Additionally, despite evidence of significant muscle damage in all studies and the sustained increase in β-CTX in Study I, there was no evidence of increased cytokine concentrations up to 4 days post-exercise. These findings do not preclude the possibility of cytokine involvement in changes in bone turnover with exercise, particularly during a period of strenuous training or where recovery is in adequate. However, based on recent data, there remain a number of questions to be answered regarding the role of changes in circulating cytokine concentrations on bone metabolism, both with disease and in response to exercise.

CHAPTER IX

GENERAL DISCUSSION

The main findings from the studies in this thesis are as follows:

1. In fasted, early morning samples, plasma β-CTX concentrations were significantly increased from 1 to 4 days following an exhaustive bout of treadmill running but were not significantly different from baseline on the recovery days in any other study. The β-CTX response to running was not affected by training status (Training Status study, Figure 4.4) and was influenced only modestly in the first hour post-exercise by increasing cardiovascular exercise intensity (Exercise Intensity study, Figure 6.4). Despite, suppressing resting, pre-exercise concentrations, the ingestion of a meal did not attenuate the increase in β-CTX with subsequent running and resulted in a more sustained increased in the early post-exercise period compared with fasting (Pre-feeding study, Figure 7.4).

2. In Study III, bone ALP, but not P1NP or OC, was significantly increased (1-7%) at 3 and 4 days post-exercise, but bone formation markers were not significantly different from baseline on the four recovery days in any other study. P1NP was increased (10-30%) during exercise, but this increase was not affected by cardiovascular exercise intensity (Exercise Intensity study, Figure 6.4) or pre-feeding (Pre-feeding study, Figure 7.4).

3. When measured in SMV samples and corrected for Cr concentrations, fPYD and fDPD did not reflect the increase in β-CTX in Study I and were significantly reduced at 2 days post-exercise. There were no significant changes in Cr-corrected pyridinoline concentrations at individual time points from 1 to 4 days post-exercise, in any other study. Cr concentrations were increased more than 300% at 1 to 2 days post-exercise in Study I and increased 100% at 1 days post-exercise in Study II but were not significantly different from baseline on the four recovery days in the Exercise Intensity and Pre-feeding studies (Figures 6.6 and 7.6). There was considerable intra- and inter-individual variation in the Cr response to exercise (All studies, Figures 4.7, 5.7, 6.7 and 7.7) as well as in Cr concentrations measured on consecutive days from rested subjects (Training Status study, Figure 4.7).

4. Mean fPYD and fDPD output, calculated from free pyridinoline concentrations multiplied by SMV urine volume, was significantly reduced (20%) at 4 days after two, 60 min bouts of running at 65% VO_{2max} separated by either 23 h or 3 h, although there was no effect of recovery duration (Recovery Duration study, Figure 5.8). There was no significant change in pyridinoline output from 1 to 4 days post exercise in the Exercise Intensity study (Figure 6.8) while output was significantly increased at 4 days in the Pre-feeding study (Figure 7.8), although there was no effect of pre-feeding. Mean urine volume was significantly reduced (25-40%) at 1 day

post-exercise in the Recovery Duration study (Figure 5.8) but was not significantly different from baseline at 1 to 4 days post-exercise in the Exercise Intensity study (Figure 6.8) or the Pre-feeding study (Figure 7.8). There was considerable intra- and inter-individual variation in SMV urine volume in samples collected on consecutive day in exercising subjects (Recovery Duration, Exercise Intensity and Pre-feeding studies, Figures 5.9, 6.9 and 7.9).

5. OPG concentrations were generally increased by acute exercise (All studies, Figures 4.8, 5.10, 5.11, 6.10 and 7.10) but only following exhaustive exercise did this increase persist until 24 h post-exercise (Training Status study, Figure 4.8). The OPG response was not affected by improved training status (Training Status study, Figure 4.8), reduced recovery duration (Recovery Duration study, Figures 5.10 and 5.11), cardiovascular exercise intensity (Exercise Intensity study Figure 6.10) or pre-feeding (Pre-feeding study, Figure 7.10).

6. PTH concentrations were generally increased by acute running. The increase was both rapid, occurring within 20 min (Training Status and Exercise Intensity studies, Figures 4.9 and 6.11) and transient, with concentrations no longer significantly different from baseline by 30 min post-exercise (Training Status and Exercise Intensity Studies, Figures 4.9 and 6.11). The PTH response was not affected by improved training status (Training Status study, Figure 4.9), reduced recovery duration (Recovery Duration study, Figures 5.12 and 5.13), or pre-feeding (Pre-feeding study, Figure 7.11). However, when cardiovascular exercise intensity was increased from 55% to 75% VO_{2max} during 60 min of running, PTH concentrations were only increased at 75% VO_{2max} (Exercise Intensity study, Figure 6.11). Concentrations were significantly reduced (10-35%) from baseline in the first few hours post-exercise in the Training Status, Recovery Duration and Exercise Intensity studies (Figures 4.9, 5.13 and 6.11) but not in the Pre-feeding study (Figure 7.11).

7. With acute running, ACa concentrations were increased in the Training Status, Recovery Duration and Exercise Intensity studies (Figures 4.9, 5.12, 5.13 and 6.11) and unchanged in the Pre-feeding study (Figure 7.11). This increase tended to be greater with improved training status during and following exhaustive running (Training Status study, Figure 4.9), but was unaffected by reduced recovery duration (Recovery Duration study, Figures 5.12 and 5.13) or increasing cardiovascular exercise intensity (Exercise Intensity study, Figure 7.11).

8. PO_4 concentrations were increased during all bouts of acute running in all four studies. The effect was rapid, with increased concentrations after 20 min of exercise (Training Status and Exercise Intensity studies, Figures 4.9 and 6.11). This increase was not affected by improved training status (Training Status study, Figure 4.9), reduced recovery duration (Recovery Duration study, Figures 5.12 and 5.13), cardiovascular exercise intensity (Exercise Intensity study, Figure 6.11) or pre-feeding (Pre-feeding study, Figure 7.11). The increase in PO_4 during

exercise was not significantly correlated with the increase in PTH in the Training Status, Exercise Intensity and Pre-feeding studies (Figures 4.10, 6.12 and 7.12), while in the Recovery Duration study (Figure 5.14), significant and borderline significant correlations were no longer evident when outliers were removed. In the first hours post-exercise, PO_4 concentrations remained increased by 10-15% in the Training Status study (Figure 4.9), returned to baseline in the Recovery Duration and Pre-feeding studies (Figures 5.13 and 7.11) and decreased by 7-17% in the Exercise Intensity study (Figure 6.11).

9. Acute running was associated with either transiently increased (Training Status study, Recovery Duration study and Exercise Intensity studies, Figures 8.1, 8.3, 8.4 and 8.6) or unchanged (Pre-feeding study, Figure 8.8) TNF-α and IL-1β concentrations, and marked increases in IL-6 (All studies, Figures 8.1, 8.3, 8.4, 8.6 and 8.8). The magnitude of the increase in IL-6 was greatest with exhaustive exercise (Training Status study, Figure 8.1), increased with increasing cardiovascular exercise intensity (Exercise Intensity study, Figure 8.6) and appeared to increase with reduced recovery duration (Recovery duration study, Figure 8.4). All bouts of running were followed by a marked increase in IL-1ra that peaked in the first few hours post-exercise (All studies, Figures 8.1, 8.4, 8.6 and 8.8). There were marked increases in CK concentrations following all exercise protocols (All studies, Figures 8.2, 8.5, 8.7 and 8.9) but, with the exception of IL-6 that was increased at 24 post-exercise after exhaustive exercise (Training Status study, Figure 8.1), cytokine concentrations were not significantly different from baseline on the four follow-up days (All studies, Figures 8.1, 8.4, 8.6 and 8.8).

9.1 The effects of acute running on bone turnover markers

9.1.1 Plasma β-CTX

9.1.1.1 The time course of the plasma β-CTX response to acute running

The current studies are the first to show the plasma β-CTX response to acute running, measured during exercise and up to four days post-exercise. When β-CTX data from Studies III and IV are examined in combination with data from several previous studies of acute, endurance cycling (Guillemant *et al.*, 2004; Maïmoun *et al.*, 2006), it is possible to describe the early time course of this response. Figure 9.1 shows the percentage change in β-CTX with acute exercise calculated in relation to a sample taken immediately before exercise, rather than the 'baseline' sample which, in the case of Study IV, was taken approximately 2 h before exercise and, in the study of Guillemant *et al.* (2004), 1 h beforehand. Displayed in this way, the effect of acute endurance exercise alone can be isolated and described, and some novel information about factors that affect the β-CTX response are revealed.

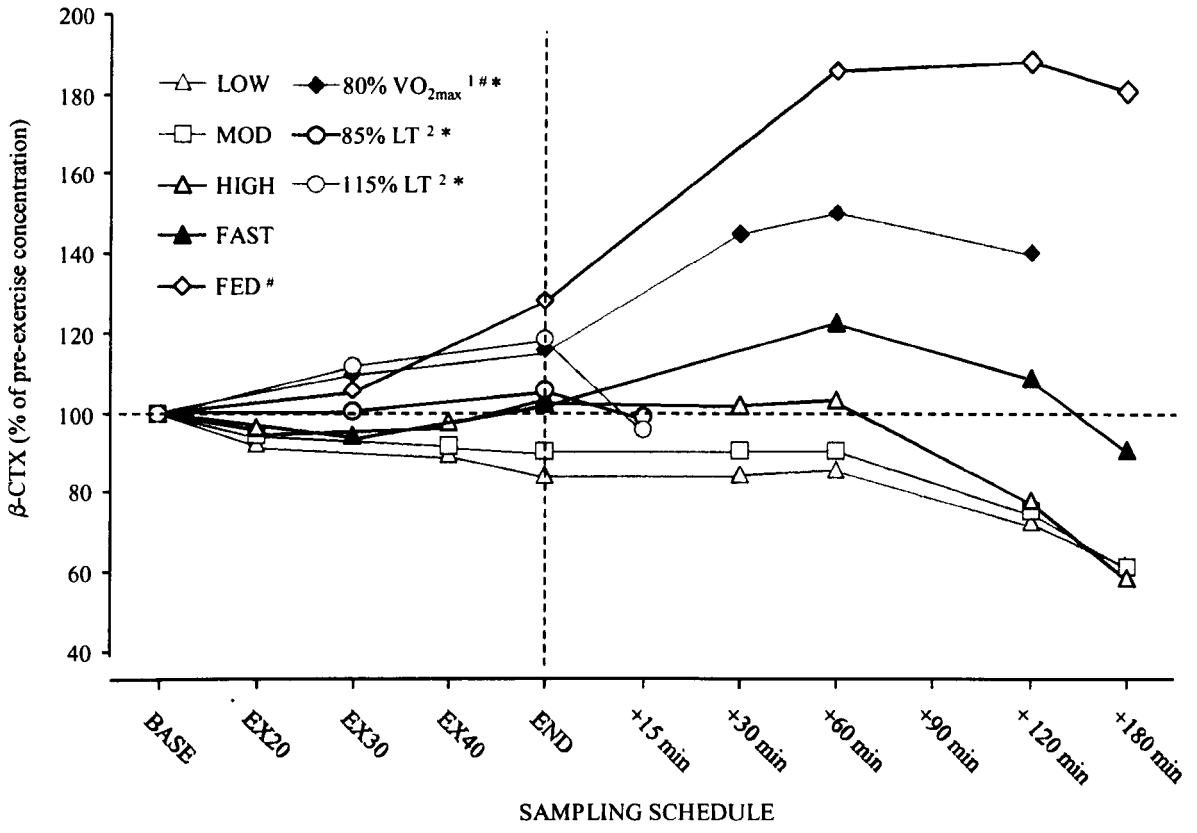

Figure 9.1. The percentage change (compared with a sample taken immediately before exercise) in β-CTX during (EX20 to EX40) and up to 3 h after (END to + 180 min) acute exercise lasting 50 or 60 min. [1] 60 min (Guillemant *et al.*, 2004); [2] 50 min (Maïmoun *et al.*, 2006). [#] subjects fed prior to exercise; * Data estimated from a published figure.

Looking specifically at the FED condition in Study IV, where subjects ate a meal prior to exercise, the pattern of change in β-CTX is in good agreement with that shown by Guillemant *et al.* (2004), with the increase in β-CTX beginning during exercise but the majority occurring in the first hour post-exercise (Figure 9.1). A similar pattern of change is also evident in the FAST condition although with a reduced magnitude. In both conditions, the magnitude of the increase exceeded the CV of the assay suggesting that it is not attributable to analytical variation. Additionally, although the magnitude is much reduced, it might also be argued that this pattern can be seen in the HIGH condition in Study III. However, this should be interpreted with caution as the magnitude of the change did not exceed the assay CV. As the duration of exercise in all studies was 60 min, it cannot be determined if β-CTX concentrations peak at 30-60 min post-exercise (*i.e.* associated with the termination of exercise) or if they peak 90-120 min after the *onset* of exercise, irrespective of exercise duration. This is an important distinction, for if the latter is true, with exercise lasting more than 60 min, the duration of the increase in β-CTX might be enhanced, as might its magnitude. Additionally, if the former is correct, it is possible that the magnitude and/or the duration of the post-exercise response might also

be enhanced with increased exercise duration. These questions will only be answered by a well-controlled study comparing 60 min of endurance exercise to that of longer duration.

9.1.1.2 Factors that affect the plasma β-CTX response to acute running

Although the findings from this thesis support those of Guillemant *et al.* (2004) regarding the short-term time course of changes in β-CTX, there are several factors that appear to affect the magnitude, and even the direction of this response. In the MOD condition in Study III, compared to 0800 h, β-CTX concentrations were decreased by 10% during and up to 1 h post-exercise (approximately 1015 h) – which , due to its magnitude, might reflect analytical variation – and by 40% by 3 h post-exercise (approximately 1215 h) (Figure 9.1). When the percentage change in β-CTX in the FAST condition in Study IV is calculated over the same time period (immediately pre-exercise [1015 h] to 1 h post-exercise [1230 h]), mean concentrations were increased 22%. However, compared to levels measured at 0800 h, they remained approximately 10% lower (Table 9.1). As the only difference between the experimental conditions for the two data sets was the time of day at which exercise commenced (1015 h *vs* 0815 h), the findings suggest that when a bout of endurance running is performed in the late morning by fasted individuals and the measurement period is standardised, the timing of exercise can affect both the magnitude and direction of the measured change in β-CTX. It also indicates that timing of the 'baseline' blood sample can have a significant effect on the calculated change in β-CTX with subsequent exercise (Table 9.1).

Table 9.1 The relative change in β-CTX in Studies III and IV and previously published literature when calculated from the 'baseline' sample or one taken immediately prior to exercise, up to 1 h post-exercise.

Study	Exercise Intensity	Pre-exercise nutritional status	Time of 'baseline' fasted sample	Time of 'pre-exercise' sample	Start time of exercise	β-CTX * change from 'baseline'	β-CTX * change from 'pre-exercise'
Study III	55% VO_{2max}	FAST	0800 h	0800 h	0815 h	-	-15%
Study III	65% VO_{2max}	FAST	0800 h	0800 h	0815 h	-	-10%
Study III	75% VO_{2maxv}	FAST	0800 h	0800 h	0815 h	-	+3%
Study IV	65% VO_{2max}	FAST	0800 h	1015 h	1030 h	-10%	+22%
Study IV	65% VO_{2max}	FED	0800 h	1015 h	1030 h	-5%	+86%
Guillemant *et al.* (2004)	80% VO_{2max}	FED	-	0930 h	0930 h	-	+45-50%

FAST, overnight fasted; FED, standardised breakfast eaten before exercise; * calculated at 1 h post-exercise.

From a methodological perspective, the latter point might be considered of little practical importance as in many studies exercise typically commences soon after a baseline blood sample is collected. However, as β-CTX concentrations decline rapidly in the late morning from their early morning peak (Wichers *et al.*, 1999), it does highlight the importance of tight standardisation of the timing of the 'baseline' sample as well as minimising the length of time between this sample and the onset of exercise if the true effect of exercise alone is to be assessed.

Examining the data from the MOD condition in Study III, together with that from the FAST and FED conditions in Study IV, gives a novel insight into how alterations in the timing of blood samples and of exercise itself, as well as the ingestion of food can affect the calculated change in β-CTX. Although the time period covered is narrow, and the number of different data sets is low, these data suggest that not only does the magnitude and the direction of the β-CTX response to 60 min of running at 65% VO_{2max} in fasted subjects depend on the time of day that exercise commences, but also that pre-exercise feeding enhances the relative increase in β-CTX (if there is sufficient time for feeding to suppress β-CTX before exercise commences). However, as with the apparent time of day effect, this difference is not evident when measured from a fasted 'baseline' sample at 0800 h (Table 9.1). As feeding suppresses β-CTX concentrations by approximately 50%, had Guillemant *et al.* (2004) compared their post-exercise β-CTX concentrations to those in a fasted sample taken before their standardised breakfast, as in Study IV, the calculated change in β-CTX would likely have been very different. Therefore, interactions between the timing of a 'baseline' blood sample, the timing of exercise and the nutritional status of subjects prior to exercise, might substantially affect how the effects of acute running on β-CTX are interpreted.

When examined in conjunction with the results of Study IV, the finding from Study III that 60 min of running at 75% VO_{2max} did not increase β-CTX requires further discussion. Firstly, in the FAST condition in Study IV, β-CTX concentrations were decreased 25% by 1015 h. This suggests that, had a non-exercising control group been included in Study III, it is likely that β-CTX concentrations would have been significantly higher than controls at 1 h post-exercise, particularly in HIGH, where concentrations were 103% of BASE levels. Secondly, as β-CTX was increased with exercise at 65% VO_{2max} in the FED condition in Study IV, and the magnitude of the change in β-CTX in LOW in Study III was similar to that in MOD, it is possible that 60 min of running at 55% VO_{2max} in pre-fed subjects might also increase β-CTX. Finally, as β-CTX concentrations in HIGH were higher than MOD and LOW in the first hour post-exercise in Study III, it is also possible that acute running at 75% VO_{2max} performed in the fed state might result in a markedly greater increase in β-CTX than that observed at 65% VO_{2max} in Study IV. Based on these data, any effect of exercise intensity on β-CTX might well also be more prominent in pre-fed subjects.

Combining the findings of Guillemant *et al.* (2004) with data from Study IV allows the first meaningful (albeit still indirect) comparison of β-CTX responses to running and cycling in fed subjects. This comparison indicates a larger relative increase in β-CTX with running, despite a 15% lower exercise intensity (Figure 9.1 and Table 9.1). Although the time of day of the pre-exercise meal (0815 *vs* 0600-0630) and the length of time between feeding and exercise (2.25 h *vs* 3-3.5 h) were different, this might suggest that running (or weight-bearing exercise) is more potent than cycling at stimulating bone resorption. Surprisingly, the current literature lacks a well-controlled, direct comparison of the bone turnover marker response to different modes of exercise and this remains an important area for future investigations.

9.1.1.3 The influence of the plasma β-CTX circadian rhythm on the response to acute running

The apparent complexity of the relationship between the start time of exercise, the timing of the 'baseline' blood sample, pre-exercise nutritional status and the β-CTX response to running, might be explained by its circadian rhythm and the influence of food intake on this rhythm. When initiated in the mid to late morning, the effects of any intervention with the potential to increase β-CTX are overlaid on to a background of declining concentrations, which might attenuate the potency of the intervention. Evidence of the influence of the circadian rhythm can be seen in Study III and the FAST condition in Study IV as, irrespective of β-CTX concentrations at 1 h post-exercise (103% to 85% of 0800 h values) by 3 h post-exercise, concentrations were reduced to <70% of 0800 h values in all groups. This decrease cannot be attributable to analytical variation as its magnitude exceeded the assay CV and is comparable with that seen in rested, fasting subjects in the late morning (Christgau, 2000).

The larger relative increase in β-CTX observed in fasted subjects when the start time of exercise was delayed to 1015 h also might be explained by the circadian rhythm, as the rate at which basal β-CTX concentrations decline lessens in the late morning, possibly lessening the potency of the rhythm to attenuate the exercise response. As discussed above, this apparently small shift in the timing of exercise appears sufficient to alter the direction of change in β-CTX with exercise at 65% VO_{2max} and produce a greater increase at 65% VO_{2max} compared with exercise at 75% VO_{2max}, further highlighting the potency of the circadian rhythm.

The effect of food ingestion on the circadian rhythm of β-CTX in the late morning might also explain why the largest relative increase was seen in the FED condition in Study IV. Feeding at around 0800 h enhances not only the magnitude of the morning decline in β-CTX, but also the rate at which it occurs, with the nadir occurring by 1100 h (Christgau, 2000; Bjarnason *et al.*, 2002; Qvist *et al.*, 2002). In fed subjects therefore, any attenuation of the stimulatory effects of running due to the circadian rhythm might already have been eliminated. Thus, it is possible that the closer to the nadir

of the β-CTX circadian rhythm – which is different with fasting and feeding – the greater the relative increase in β-CTX with acute running that will be observed.

Despite the evidence of the circadian rhythm of β-CTX in fasted subjects in the first 3 h post-running, any influence of the rhythm apparently disappears with pre-exercise feeding, with concentrations in the early afternoon close to those seen at 0800 h, instead of the marked decrease that would be expected by this time with feeding alone (Qvist *et al.*, 2002). Again, had Guillemant *et al.* (2004) taken a measure of β-CTX prior to feeding their subjects and included a rested control group for comparison, it is likely that they would have observed a similar pattern of change. Thus, the data from Study IV seems to confirm a novel interaction between pre-feeding and acute exercise.

As no measures were taken between 3 h and 24 h post-exercise in Study IV, the exact duration of the increase in β-CTX cannot be determined. In rested subjects fed at around 0800 h, β-CTX concentrations begin to increase around 1400 h (6 h later) and are similar to those in fasted subjects at about 1700 h (9 h later), although concentrations remain considerably lower than those seen at 0800 h at this point and remain so until late in the evening (Christgau, 2000; Bjarnason *et al.*, 2002; Henriksen *et al.*, 2003). As β-CTX concentrations in the FED condition were already within 10% of 0800 h levels by 1 h post-exercise (1230 h), this might indicate that acute running shortens the duration of the suppression of β-CTX by feeding and also increases concentrations to a level above that that would be expected in rested individuals, at least during the day and evening. If so, it is possible that the overall effect of a single bout of acute, endurance running in fed subjects might be to increase the mean 24 h β-CTX concentration compared with resting. Again, well-controlled studies with rested controls groups (both fed and fasted) are needed to fully explore the magnitude, and thus the significance, of post-exercise changes in β-CTX.

9.1.1.4 Changes in plasma β-CTX with acute running from 24 h post-exercise and beyond

Of all the exercise protocols studied, only intermittent, exhaustive running in Study I increased β-CTX on the four recovery days. The magnitude of the increase on these days exceeded the CV of the assay suggesting that it is not attributable to analytical variation. Interpreting previous studies that have reported increases in β-CTX on the days following exercise is limited by their failure to tightly standardise the time of day of blood sampling and the nutritional status of subjects prior to sampling, and the lack of a non-exercising control group (Herrmann *et al.*, 2007; Kerschan-Schindl *et al.*, 2009). However, with the standardisation of the time of day of blood sampling, an overnight fast prior to all samples being collected, and the inclusion of a control group, the results of Study I confirm that acute, strenuous, endurance exercise can elevate β-CTX concentrations for up to 4 days.

The failure of Study II to elevate β-CTX concentrations on any of the four recovery days, particularly in the SHORT condition, was surprising and Studies III and IV attempted to investigate the role of two factors, exercise intensity and pre-exercise nutritional status, which might have contributed to these

different responses. The results of these studies suggest that neither factor can adequately account for the different β-CTX responses and that other factors must be involved. The lack of any change in β-CTX in Study II is unlikely to be because two bouts of running had no effect at all on β-CTX as, in Study IV, β-CTX concentrations were increased by a single, 60 min bout of running at 65% VO_{2max}. However, whatever the magnitude or duration of the response, it was not sustained through to the recovery days.

The possible role of changes in PTH in the different β-CTX responses is discussed in Section 9.3.3, but a further speculative possibility is that the sustained increase in β-CTX in Study I might, in part, be related to the metabolic demand of the exercise protocol and its short-term effect on energy availability. Severely reducing energy availability through a combination of exercise and caloric restriction increases basal β-CTX concentrations (Ihle and Loucks, 2004), with the increase coinciding with a decrease in estradiol, luteinising hormone (LH) pulse frequency, and blood glucose, the latter of which decreases by 0.7 $mmol \cdot L^{-1}$ or 15% (Loucks and Thuma, 2003). Glucose is a key regulator of gonadotropin releasing hormone, and thereby LH pulsatility (Ohkura *et al.*, 2004) and as blood glucose was reduced by 20-25% with exhaustive running, it is possible that a short-term reduction in energy availability induced by exercise, possibly in combination with the effects of a 17 h fast, contributed to the sustained increase in β-CTX observed only in Study I. Future interventional studies that protect blood glucose levels (*e.g.* through carbohydrate ingestion) during exhaustive exercise might allow this hypothesis to be tested. As reductions in blood glucose are more frequently observed with acute cycling, this mode of exercise might be used to investigate this mechanism in a more repeatable and controlled manner.

9.1.2 The effects of acute running on bone formation markers

Taken collectively, the studies presented in this thesis show, for the first time that, under controlled conditions that standardise both the time-of-day of blood sampling and the nutritional status of subjects, there is little effect of acute, endurance running on bone formation markers, at least up to 4 days post-exercise. Importantly, the physiological demands of the protocols investigated range from moderate (60 min at 55% VO_{2max}), which might be undertaken by a recreationally-active individual, to strenuous (60 min at 75% VO_{2max}) and repeated (Study II), which might represent an athletic or military training session, up to long-duration and exhaustive (Study I), possibly representative of a competitive sports event or a military exercise.

Although the current studies are the first to measure the P1NP response to acute, endurance running, findings from them differ from previous investigations of cycling (Herrmann *et al.*, 2007; Pomerants *et al.*, 2008) and walking (Tosun *et al.*, 2006) in that P1NP was increased during exercise but was not decreased at 3 h and 24 h post-exercise. Although further investigations are required to confirm the

findings from the current studies, the superior degree of experimental control employed by the current studies might, in part, explain these differences.

Studies III and IV are the first investigations to show that P1NP is increased during endurance running. As basal P1NP concentrations decrease in the late morning (Ahmad *et al.*, 2003) and the magnitude of the increase with exercise (10-41%) across the two studies exceeds the CV of the assay, together points to a genuine effect of exercise. The increase during exercise was progressive, transient in nature and, at least in Study IV, appeared to precede any increase in β-CTX. Although it cannot be completely excluded, based on this evidence, it is difficult to conclude that this represents a genuine increase in type 1 collagen formation in bone. However, as P1NP is considered a specific marker of bone formation, it is important to understand what this increase might reflect.

As discussed previously, with P1NP being cleared via scavenger liver receptors (Melkko *et al.*, 1994), the increase is unlikely to be explained by the accumulation of P1NP in the circulation due to changes in kidney function during exercise. P1NP is increased in the interstitial fluid surrounding both tendon and muscle tissue in the hours following exercise (Crameri *et al.*, 2004; Miller *et al.*, 2007; Hansen *et al.*, 2008) but there is no evidence of a concomitant increase in P1NP in the circulation (Miller *et al.*, 2005). Collagen metabolism has not been directly measured in muscle and tendinous tissue during running and compared to changes in serum P1NP, but it is logical that changes in tissue collagen metabolism would precede an increase in P1NP in the circulation. Therefore, the increase in circulating P1NP during exercise in Studies III and IV seems unlikely to reflect the acceleration in type 1 collagen metabolism in these tissues.

There are three possible alternative explanations. Firstly, increased blood flow through bone due to exercise (Fellman, 1992) might result in the accelerated appearance of P1NP in the circulation. Secondly, as suggested by Brahm *et al.* (1997b), microdamage to the skeleton could lead to a release of substances including P1NP previously trapped in bone. Finally, as P1NP is a relatively large (27 kDa) molecule (Jensen *et al.*, 1998), the increase in P1NP might reflect the retention of P1NP already present in the circulation when plasma water moves into the extravascular space as a result of exercise. The latter theory is supported by an increase in the concentration but not the content (concentration corrected for hemoconcentration) of P1CP, a large (100 kDa) molecule, during exercise (Brahm *et al.*, 1997a). Any explanation must also account for lack of any increase in OC in Studies III and IV over the same time period.

All three theories might explain the tendency for the increase in P1NP to be greater with increasing exercise intensity in Study III, while the blood flow and hemoconcentration theories might also best account for the rapid resolution of P1NP concentrations with the termination of exercise. As P1NP is cleaved off and released by collagen metabolism during formation, but OC is tightly bound to the bone matrix, the blood flow (and to a lesser extent the microdamage) theory might best explain the lack of

any change in OC although, as OC is a relatively small (5.8 kDa) molecule (Eriksen *et al.*, 1995), the hemoconcentration might also account for this.

As bone ALP is localised in the plasma membrane of osteoclasts and is larger (68 kDa) than P1NP (Panigrahi *et al.*, 1994), an increase in its concentrations during exercise is unlikely to be accounted for by the blood flow and damage theories, but would be consistent with a hemoconcentration effect. Bone ALP was not measured during exercise in Studies III and IV and there are inconsistent findings from studies of acute, endurance cycling (Guillemant *et al.*, 2004; Maïmoun *et al.*, 2006). In the study of Guillemant *et al.* (2004), bone ALP levels were not increased during 60 min of cycling at 80% VO_{2max}, despite a marked hemoconcentration. As the change in P1NP (up to 30%) in Studies III and IV was far greater than might be expected due to hemoconcentration alone, together with the lack of any change in bone ALP in the study of Guillemant *et al.* (2004), this suggests that an exercise-associated hemoconcentration is unlikely to explain the increased P1NP.

In summary, if the transient increase in P1NP during exercise in Studies III and IV is not due to the formation of type 1 collagen in bone, based on the available data, an acceleration of the appearance of P1NP into the circulation due to an increase in blood flow through bone appears to be the most likely cause. However, as an increasing number of studies indicate that bone formation markers can respond in a matter of minutes to hours to a range of physiological stimuli (Clowes *et al.*, 2002b; Clowes *et al.*, 2003; Henriksen *et al.*, 2003), the possibility that the increase in P1NP does indeed reflect new bone formation cannot be excluded completely. Until more data is available, this cannot be confirmed and any explanation for the increase in P1NP during exercise remains only speculative.

The only marker of bone formation to show any change on the four follow-up days in the current studies was bone ALP in Study III, which was increased on follow-up days 3 and 4. Thus, Study III is the first study to report an increase in bone ALP in the days following acute exercise, with previous studies showing either decreased concentrations (Ashizawa *et al.*, 1998; Mouzopoulos *et al.*, 2007) or no effect of exercise (Malm *et al.*, 1993; Brahm *et al.*, 1996; Welsh *et al.*, 1997; Whipple *et al.*, 2004). This finding should be interpreted with caution, however, as the magnitude of the change (1-7%) across the three conditions falls within the CV of the assay. Of P1NP and bone ALP, bone ALP might be considered to have been the less likely to respond in the time period of the current studies, as it reflects the mineralisation of the bone matrix, which should occur subsequent to the formation of the matrix itself. It is possible that the increase in bone ALP might reflect an increase in the mineralising activity of already active osteoblasts and three days of increased cellular activity are required before this activity is evident in changes in circulating bone ALP concentrations. Indeed, recently it has been shown that, like P1NP, bone ALP is significantly increased after only 3 days of PTH therapy (Glover *et al.*, 2009) suggesting a rapid increase in mineralisation coincident with the formation of new collagen matrix as part of the anabolic response.

An important question arising from the current studies is why bone ALP was increased in Study III and not in the other studies, particularly as the research design in Study IV is comparable, while in Study II there is potentially twice the exercise stimulus. The completion of exercise at 75% VO_{2max} is unlikely to explain this as there was no effect of exercise intensity on bone ALP responses and, if anything, the increase in bone ALP tended to be greater with exercise at 65% VO_{2max} (6% *vs* 4%). Importantly, Study I is the first study to measure bone ALP on the days following acute exercise and include a non-exercising control group. Thus, whether the increase in bone ALP in Study III would be significant compared to a rested control group requires confirmation in future studies.

The general lack of any marked effects on bone formation markers with the various running protocols employed in the current studies might be explained in several ways. Firstly, one or two bouts of running might have no effect on bone formation. Given the duration and intensity of the bouts of running, and evidence of anabolic activities ranging from an increase in c-fos mRNA (Raab-Cullen *et al.*, 1994) to the formation of new bone (Forwood *et al.*, 1996) after a single loading session, this explanation seems unlikely.

Alternatively, it might be that running induced an anabolic response at the cellular level that was not sufficient to result in a change in bone formation markers, or that the follow-up period of the studies was simply insufficient to detect the bone formation response. Following 4 weeks of jumping training, no change in serum ALP or OC is reported in C57BL/6J (B6) mice, despite increases in structural bone formation parameters (Kodama *et al.*, 2000), suggesting exercise can have local anabolic effects that are not evident at a systemic level. Until animal studies are performed in which cellular events, bone formation parameters and serum bone formation markers are measured following a single bout of loading, if, or when, a change in bone formation markers might occur with acute exercise remains unknown. Whether increases in bone formation markers occur more than 4 days after acute running can only be answered by longer studies. However, the current studies are a significant step forward by showing no increase up to 4 days, whilst making concerted efforts to control for the potential confounding factors of circadian rhythms, pre-sample nutritional status, non-prescribed exercise and energy balance. The challenge for future studies will be to extend the follow-up period whilst maintaining sufficient control of these factors.

9.1.3 Urinary pyridinolines in the measurement of changes in bone resorption with acute running

The current studies are the first to systematically investigate the effects of acute running on the urinary pyridinolines. Across all studies the magnitude and direction of changes in fPYD were identical to those in fDPD which is consistent with the only previous study of acute exercise to measure both (Welsh *et al.*, 1997). The magnitudes of changes were small and generally within the CV of the assays, and taken together with those of Welsh *et al.* (1997), the results from this small group of

studies provides no evidence of a selective effect of a range of exercise protocols on either of these degradation products of type 1 collagen.

Importantly, these studies are also the first to measure both fDPD and β-CTX in response to the same exercise protocols allowing a comparison between two markers considered specific for bone resorption. A key finding from these studies is that in contrast to β-CTX, fPYD/Cr and fDPD/Cr were not significantly increased following exhaustive exercise (Figure 9.2) and were, in fact, significantly reduced at two days post-exercise. As β-CTX is considered a specific marker of bone resorption, and the necessity to use a biochemical correction factor for urinary markers introduces additional variability, in this instance, it might be appropriate to consider β-CTX the 'gold standard' against which other biochemical measures of bone resorption are compared. It could thus be concluded that fPYD/Cr and fDPD/Cr fail to reflect the increase in bone resorption associated with exhaustive running.

Given the marked increase in Cr following exercise, an obvious question is what role this increase had in the fPYD/Cr and fDPD/Cr responses. Although some increase in mean Cr concentrations was expected, the increase in Study I was greater than that previously reported in the literature, most likely reflecting a large increase in the turnover of muscle creatine with exercise. Logically, an increase in Cr of a greater magnitude than any in the pyridinolines might result in a lowering of the Cr-corrected pyridinoline concentrations. However, although increased Cr might, in part, have influenced the fPYD/Cr and fDPD/Cr responses, with no significant decrease in fPYD/Cr and fDPD/Cr at FU1 when mean Cr increases were greatest (Figure 9.2), and decreased pyridinolines also in the CON group at FU2, this appears unlikely to fully explain the different findings for fPYD/Cr and fDPD/Cr compared with β-CTX.

As an alternative (and comparison) to correcting for Cr concentrations, pyridinolines were also expressed in terms of their 'output' in Studies II-IV, by multiplying pyridinoline concentrations by the SMV urine volume (Figure 9.3). Calculating pyridinoline output has previously only been performed from 24 h urine samples so there is no precedent for calculating total pyridinolines from SMV samples. However, as collecting a SMV is likely a more reliable method than a 24 h collection in studies of free-living individuals, and running significantly affected Cr concentrations in Study I, it was of interest to see how this method might compare to Cr-corrected results from the same SMV sample.

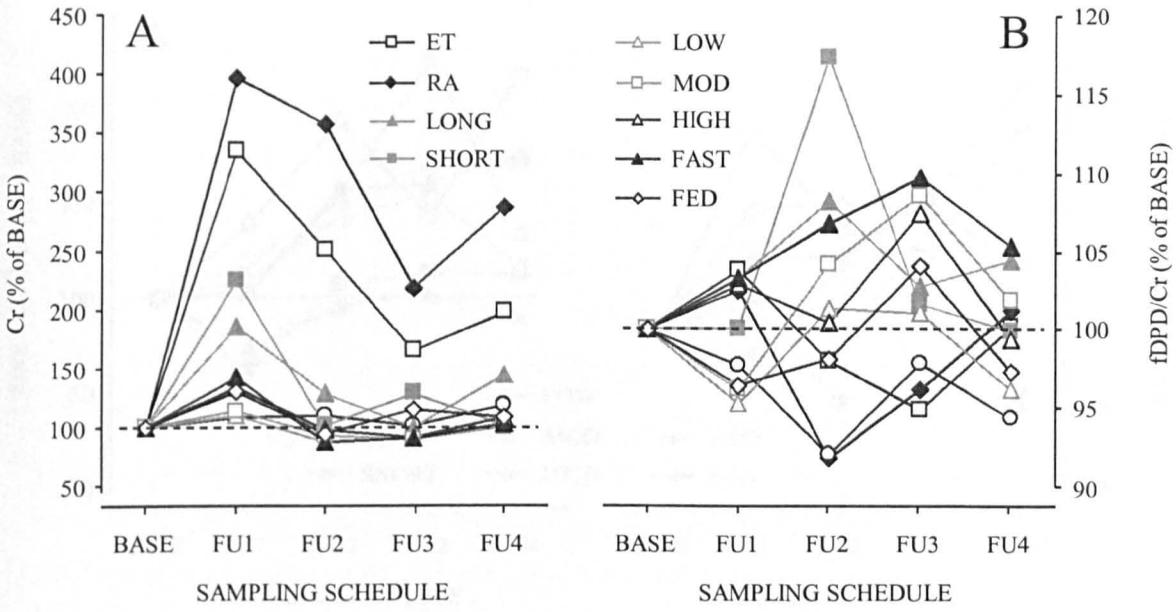

Figure 9.2 Percentage change from baseline (BASE) on the four follow-up days (FU1 – FU4) in Cr (Panel A) and fDPD/Cr (Panel B) in Studies I-IV. As there were no significant differences between fPYD/Cr and fDPD/Cr during any study, only results for fDPD/Cr are shown.

Unlike in Study I, there was no increase in β-CTX on the four follow-up days in Studies II to IV with which to compare the pyridinoline output and Cr-corrected findings. However, only in Study III did the fPYD and fDPD output results agree with the unchanged β-CTX concentrations. Additionally, where there was a significant change in fPYD and fDPD output, the direction of this change was not consistent, with mean values at FU4 decreased in Study II but increased in Study IV. If anything, in terms of a comparison with β-CTX, Cr-corrected results in Studies II-IV appear to perform slightly better, with unchanged values in all three studies, although there was a trend for increasing values at FU3 in Study III. Certainly the increase in mean Cr concentrations following exercise in Studies II-IV was less marked than in Study I, particularly in Studies III and IV, although it was still significant at FU1 in Study II (Figure 9.2). That said, the only significant difference in mean urine volume from pre-exercise values in any study was a significant reduction in Study II at FU1.

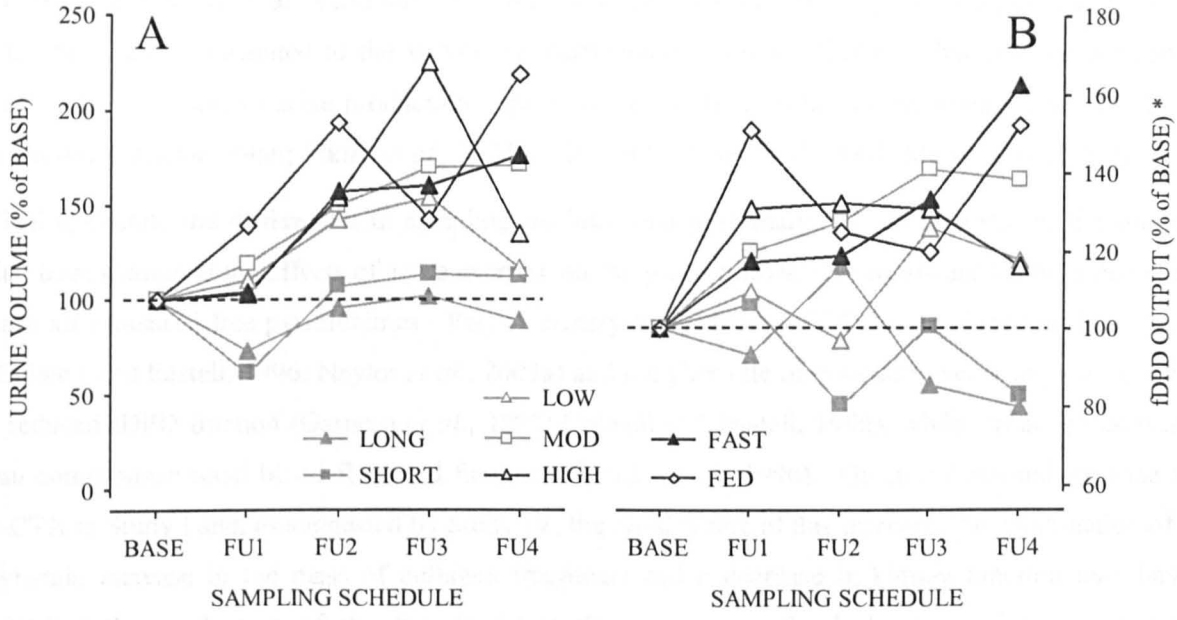

Figure 9.3 Percentage change from baseline (BASE) on the four follow-up days (FU1 – FU4) in urine volume (Panel A) and fDPD output (Panel B) in Studies II-IV. As there were no significant differences between fPYD and fDPD output during any study, only results for fDPD are shown. * fDPD output (mmol) = fDPD ($mmol \cdot L^{-1}$) x urine volume (L).

It is not possible to determine from these studies to what degree the significant changes in pyridinolines are influenced by changes in Cr and urine volume but, as discussed above, Figures 9.2 and 9.3 do not indicate that fluctuations in mean Cr-corrected pyridinolines, or pyridinoline output, consistently coincide with fluctuations in mean Cr concentrations and mean urine volumes respectively. Although there were few significant changes in mean Cr concentrations and urine volumes, an examination of individual subjects revealed considerable variability in both. Thus, when mean Cr concentrations were markedly increased in Study I, there was marked variability in the magnitude of this increase, while in the remaining studies, Cr concentrations and urine volumes were both increased and decreased on the follow-up days in different individuals, with no consistent pattern apparent. This variability explains why there were few significant changes in Cr concentrations and urine volumes despite marked changes in mean values (Figures 9.2 and 9.3). These individual variations might also partly underlie the failure of either method to accurately reflect the changes in β-CTX, with the significant differences in mean Cr-corrected and pyridinoline output values representing, in part, where fluctuations in a majority of subjects in these small groups happen to coincide.

To what degree this variability represents natural day-to-day fluctuations or the effects of exercise are unknown, but data from the CON group suggests a marked variation in day-to-day Cr concentrations in non-exercising individuals consuming a non-creatine controlled diet. In the present studies it was necessary to collect a SMV sample within a limited time frame, both to control for the potential effects

of feeding and diurnal variation and for practical reasons relating to subject availability. This may have contributed to the variability, particularly in urine volume. That said, considerable day-to-day variation in urine production appears to be inherent, even in a rigorously collected 24 h collection (Jackson, 1966; Elkins *et al.*, 1974; Duke, 1998; Jones *et al.*, 2007; Marco *et al.*, 2008).

Finally, despite the differences in sampling methods and post-analytical 'corrections' in the studies that have examined the effects of acute exercise on the pyridinolines, one consistent factor is that they have all measured free pyridinolines. Part of urinary free fraction of DPD is produced in the kidney (Colwell and Eastell, 1996; Naylor *et al.*, 2003a) and a higher rate of bone turnover is associated with a reduced fDPD fraction (Garnero *et al.*, 1995; Colwell and Eastell, 1996), while strenuous exercise can compromise renal blood flow and function (Suzuki *et al.*, 1996). Given the marked increase in β-CTX in Study I and, as suggested by Study IV, the rapid nature of this increase, the combination of a dynamic increase in the mass of collagen fragments and a decrease in kidney function may have inhibited the production of the free fragment thus explaining the lack of any increase in free pyridinolines. As total pyridinolines change more dynamically than free pyridinolines with metabolic bone disease and subsequent therapy (Blumsohn *et al.*, 1995b; Garnero *et al.*, 1995; Tobias *et al.*, 1996; Naylor *et al.*, 2003a), it is possible that total pyridinolines might also be a more sensitive measure of the bone resorption response to strenuous, acute exercise. Future studies should, therefore, measure total pyridinolines as well as the free and peptide-bound fraction. The availability of a suitably sensitive assay to measure the low serum concentrations of the pyridinolines would also provide useful information in this regard.

In summary, fPYD/Cr and fDPD/Cr concentrations did not reflect the marked increase in β-CTX following exhaustive exercise and neither Cr-corrected pyridinolines nor pyridinoline output consistently reflected the unchanged β-CTX in the remaining studies. Although the significant changes observed with both methods do not appear to result directly from significant changes in their 'correction' factors, considerable individual variability in Cr and urine volume in a SMV likely hampers the precision of each technique. Thus, from the limited data in the current studies, from a fasted, early morning SMV, neither technique appears to be useful in terms of assessing bone resorption. Although more invasive, a fasted, early morning measure of β-CTX would appear to be the preferable method for assessing bone resorption under these conditions.

9.1.4 The net effects of changes in bone resorption and formation with acute running on bone turnover and its implications

Taken together, the current studies suggest that a bout of running is associated with a rapid increase β-CTX that, if exercise strenuous and exhaustive, may persist for up to 4 days post-exercise while there is little evidence of a concomitant increase in bone formation markers. Although the magnitude and duration of the increase in β-CTX appears to be dependent on several factors, viewed in combination with previous studies of high intensity (Guillemant *et al.*, 2004) and extremely long duration (Kerschan-Schindl *et al.*, 2009) exercise, the effect of acute exercise on bone turnover appears to be reasonably consistent. Theoretically at least, an imbalance between resorption and formation favouring increased resorption would lead to a net loss of bone tissue (Garnero *et al.*, 1996). Combined with factors such as a waning of the bone formation response to long-term mechanical loading (Turner *et al.*, 1992; Kim *et al.*, 2003; Saxon *et al.*, 2005; Schriefer *et al.*, 2005) and the suppression of basal bone formation with reduced energy availability (Zanker and Swaine, 2000; Ihle and Loucks, 2004), if the increase in bone resorption in preserved with repeated exposure to acute running – as suggested indirectly by Study I – this may result in an imbalance between resorption and formation in populations participating in regular, strenuous running activity. Thus, the acute β-CTX response to endurance running, might, in part, explain reports indicating reduced spinal BMD in some endurance runners (Blinan *et al.*, 1989; Hetland *et al.*, 1993a; Hetland *et al.*, 1993b; Hetland *et al.*, 1994; Hind *et al.*, 2006).

As bone loss in conditions such as postmenopausal osteoporosis results from a continuous and prolonged imbalance in bone resorption and formation lasting months or years, a key question arising from the current studies is whether the β-CTX responses are clinically significant and thus, over time, have the potential to result in measurable changes in bone structure and quality. Although Study I indicates a sustained elevation in β-CTX with exhaustive exercise, the protocols used in the other studies might be more indicative of a regular daily exercise session. Additionally, as active populations are more likely to exercise in the non-fasted state, the β-CTX response in FED in Study IV might be considered the most ecologically valid representation of daily exercise. If so, then the typical β-CTX response to acute running appears to be a transient increase that lasts for several hours, but is no longer evident at 24 h post-exercise. As repeated suppression of the nocturnal increase in β-CTX has been shown to significantly increase BMD at the hip (Henriksen *et al.*, 2009), in principle at least, this suggests that repeated transient changes in bone resorption can, over time, lead to alterations in bone quality. Additionally, a recent study indicates that the effectiveness of pharmaceutical suppression of β-CTX appears to be related to the time of day and subjects' pre-dosing nutritional status (Karsdal *et al.*, 2009). As similar factors appear to interact in determining the measurable change in β-CTX with acute running, although only speculative at this stage, it is possible that the

effects of running on bone resorption might be enhanced or diminished depending on pre-exercise conditions.

The effects of exercise are likely less potent that those induced by pharmaceutical interventions and it is possible that a considerable period of time, possibly many years of training, must pass before any negative effects of running are detectable at the level of BMD. A lack of change in BMD with 12 months of endurance running training (Bennell *et al.*, 1997) certainly suggests that any negative effect of running is not a potent or rapid one. However, unlike running, BMD is reduced after only a single season in cyclists (Barry and Kohrt, 2008). This might indicate some form of protective effect of running, possibly due to its weight-bearing nature. As the acute β-CTX response to cycling is abolished by calcium ingestion (Guillemant *et al.*, 2004) a season-long interventional study of calcium supplementation during exercise might provide a unique opportunity to investigate the role of exercise-associated increases in bone resorption in longer-term changes in BMD in endurance athletes.

An increase in β-CTX with acute running might also provide an insight into the high incidence of stress fractures in military recruits, as increased bone resorption in response to weight bearing exercise is thought to transiently reduce both bone stiffness and strength, increasing susceptibility to further damage (Johnson *et al.*, 1963; Schaffler *et al.*, 1989; Schaffler *et al.*, 1990; Martin, 1995). Thus, while an imbalance in bone turnover favouring bone resorption may be necessary to result in a long-term decrease in BMD, an acceleration in bone resorption alone might be sufficient to increase the likelihood of SFx during a period of repeated, weight-bearing exercise. This might provide a metabolic basis for the high incidence of SFx in military populations and also explain why a peak in SFx occurs after only 3 weeks of training (Greaney *et al.*, 1983; Garcia *et al.*, 1987; Jordaan and Schwellnus, 1994; Ross and Woodward, 1994; Heir, 1998; Popovich *et al.*, 2000). The more prolonged increase in β-CTX with exhaustive exercise in Study I and the modest effect of exercise intensity seen in Study III might also partly explain why recruits with low aerobic fitness are more susceptible to SFx, as they likely experience a greater number of incidents where exercise is strenuous, exhaustive and of higher intensity.

Importantly, in relation to SFx, to what extent the increase in β-CTX relates directly to bone resorption at the skeletal site that is mechanically challenged by running, remains unknown. Similarly, whether BTM are increased more rapidly, or by a greater extent preceding SFx, compared to recruits who remain SFx free, is also yet to be determined. Additionally, despite females recruits having higher rates of SFx (Pester and Smith, 1992), β-CTX, P1NP and bone ALP are increased by the same amount in male and female recruits after 8 weeks of training, while pre-training concentrations of BTM are actually reported to be positively associated with physical fitness (Evans *et al.*, 2008). Thus, it might be the relative increase in bone resorption during training that is of significance. As SFx typically occur early in training, large prospective studies that make frequent measures of bone turnover

markers in the first weeks of training are required to provide insights into the role of changes in bone turnover in SFx development.

9.2 The effects of acute running on circulating OPG and its relationship with changes in bone turnover markers

9.2.1 The effects of acute running on circulating OPG concentrations

The current studies are the first controlled laboratory investigations to examine the acute time course of changes in OPG with exercise and also the first to examine its relationship with changes in BTM. The findings from these studies agree with those from previous investigations (Ziegler *et al.*, 2005; Brooke-Wavell *et al.*, 2007; Kerschan-Schindl *et al.*, 2009) that exercise increases OPG concentrations, but also provide new information regarding its time course and how this time course is affected by different exercise protocols (Figure 9.4). As can be seen in Figure 9.4, acute running was associated with an increase in OPG concentrations that was evident after only 20 min.

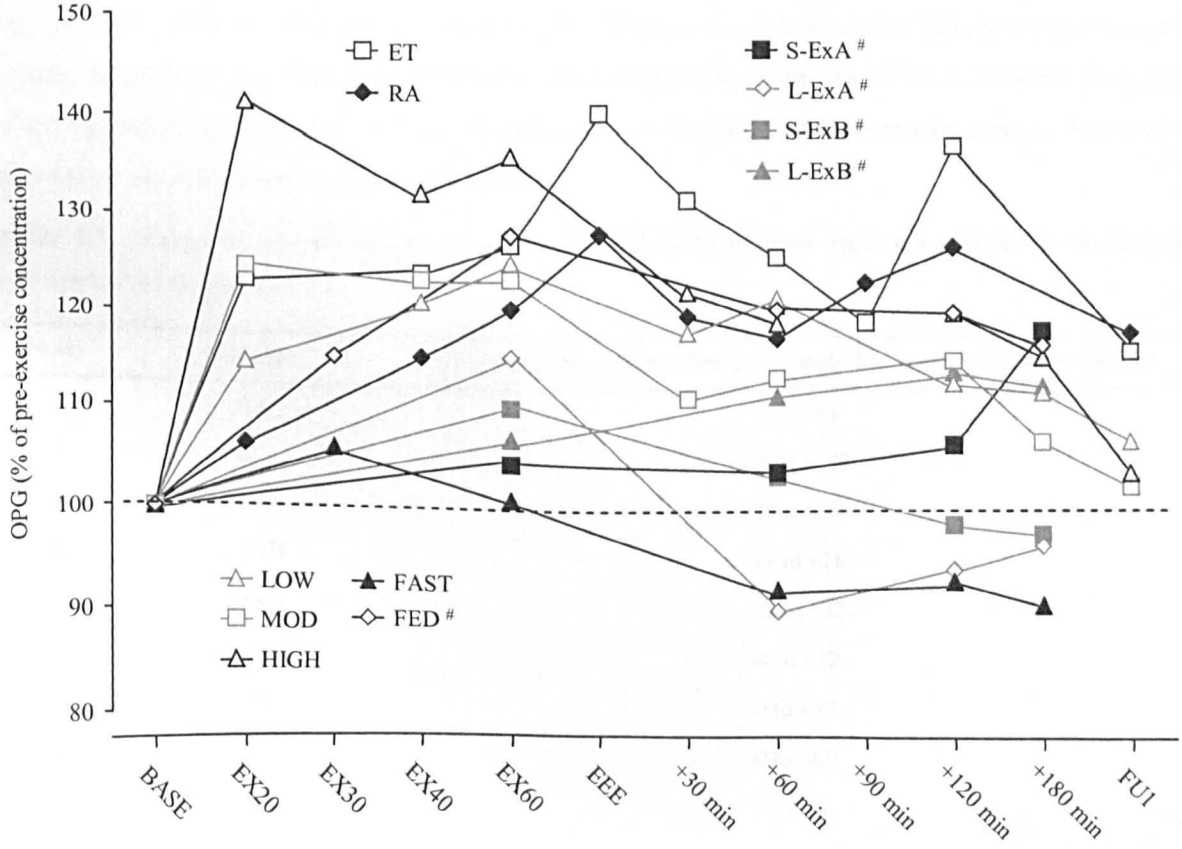

Figure 9.4. Percentage change in serum OPG concentrations from immediately pre-exercise in Studies I-IV. # subjects fed prior to exercise.

This increase was characterised either by a rapid, marked increase and a plateau thereafter, or a more progressive increase throughout exercise, apart from the FAST condition in Study IV, where only a modest and transient increase in mean concentrations was evident. There was also considerable variation in the magnitude of this response. For instance, looking only at exercise bouts lasting for 60 min at 65% VO_{2max}, the increase in mean OPG concentrations ranged from 1% (FAST in Study IV) to 28% (FED in Study IV) (Figure 9.4).

With only two other published studies to compare against (Ziegler *et al.*, 2005; Kerschan-Schindl *et al.*, 2009), neither of which measured OPG during running and both of which were performed in less controlled environments, it is difficult to explore in detail what might lie behind this variability. An examination of individual OPG responses in each study shows that these also are highly variable (Table 9.2). Some of this variability may well be analytical as the mean change in OPG concentrations in five data sets (L-ExA, L-ExB, S-ExA and S-ExB in Study II and FAST in Study IV) was less than or only slightly more than the CV (10%) of the assay. As basal OPG concentrations increase in the late morning (Joseph *et al.*, 2007) some of the variability may also be biological but not attributable to exercise. However, the magnitude of increases in OPG in other data sets were markedly greater than the assay CV and occurred over a short time scale (BASE to EX20 = 35 min). Even after allowing analytical variability and the circadian rhythm of OPG, this points to a genuine response to exercise, at least with some exercise protocols.

Table 9.2. Range of relative change in individual OPG concentrations from pre-exercise to immediately post-exercise in Studies I-IV.

Study	Data Set	OPG (range of % change from pre-exercise* to immediately post-exercise)
I	RA	-4 to +68
I	ET	-14 to +142
II	L-ExA	-29 to +64
II	L-ExB	-19 to +28
II	S-ExA	-23 to +22
II	S-ExB	-4 to +42
III	LOW	0 to +57
III	MOD	0 to +43
III	HIGH	0 to +90
IV	FAST	-22 to +32
IV	FED	+13 to +67

* the final sample taken prior to exercise commencement.

Interestingly, the variability appears to be irrespective of either exercise intensity or total 'metabolic load', as at least one subject in each group in Study I and in the HIGH condition in Study III showed no increase in OPG. Of all the exercise protocols, the most consistent response – in terms of the direction of change – was seen with FED in study IV (+13 to +67%). That said, however, experimental conditions were near identical for S-ExA in Study II (meal at 0800 h, 60 min of running at 65% VO_{2max} starting at 1030 h) yet the range of responses was very different with individual responses ranging from -23 to +22% of pre-exercise values (Table 9.2).

One notable feature of the mean OPG response in the current studies is the considerably smaller magnitude of change in concentrations compared to those reported previously with running where concentrations were increased 85% following a marathon (Ziegler *et al.*, 2005) and 150% following a 246 km run (Kerschan-Schindl *et al.*, 2009). The maximum change in OPG in the current studies was around 40%, evident both in Study I and in the HIGH condition in Study III. Although the previously published studies have used the Biomedica OPG assay, this is unlikely to explain the difference as the Biomedica and IDS assays are fundamentally the same. The difference might be related to the overall physiological strain induced by exercise, as both a marathon and a 246 km run are highly strenuous, the marathon as a consequence of both exercise intensity and duration, and the 246 km run as a consequence of extreme duration. Of the exercise protocols used in the current studies, intermittent, exhaustive exercise was by far the most demanding and, although considerably shorter and less demanding than a marathon or a 246 km run, this was the only protocol to increase OPG concentrations at 24 h post-exercise (Figure 8.4). Although the OPG response to exercise was not significantly affected by exercise intensity, the tendency for the increase in OPG to be greatest and most rapid at 75% VO_{2max} in Study III could indicate that exercise intensity, in part, contributes to the marked increase in OPG during a marathon (Ziegler *et al.*, 2005).

9.2.2 Changes in circulating OPG in relation to changes in bone turnover markers with acute running

With the identification of the RANKL/RANK/OPG axis and its role in the control of bone remodelling, a key driving force behind investigations into circulating OPG, in the field of bone physiology at least, was the possibility that is might act as an indicator of bone's metabolic activity, with early studies suggesting that OPG was increased in concert with bone resorption (Yano *et al.*, 1999). The studies described in this thesis are the first to measure both OPG and β-CTX responses to acute running under controlled laboratory conditions and the findings from Study I show some agreement with a recent study of a 246 km competitive run that reported increases in both OPG and β-CTX (Kerschan-Schindl *et al.*, 2009). The different time courses of changes in OPG and β-CTX in Study I, however, make drawing further conclusions about the nature of this relationship difficult and in this regard, Study II is not able to provide any further useful information. In Study III, despite the

tendency for a more rapid increase in OPG at 75% VO_{2max}, any interpretation here is complicated by the modest effect of exercise intensity on β-CTX concentrations. Perhaps the most useful information comes from Study IV, where despite feeding suppressing resting β-CTX concentrations and resulting in a more sustained increase in β-CTX at 2-3 h post-exercise, there was no evidence of these differences in the OPG concentration. This study suggests that, at least under these conditions, the OPG concentration does not closely reflect acute changes in bone resorption.

The lack of a close association between OPG concentrations and bone resorption would be consistent with an increasing number of both clinical and physiological studies, many of which have been published since the studies in this thesis were designed and performed. These studies have failed to observe associations between OPG and bone resorption in cross-sectional studies of patients with bone disease (Ueland *et al.*, 2001; Han *et al.*, 2005; Kim *et al.*, 2008b) and following antiresorptive therapy (Dobnig *et al.*, 2006; Anastasilakis *et al.*, 2008a; Anastasilakis *et al.*, 2008b; Choi *et al.*, 2008; Dundar *et al.*, 2009), while physiological interventions known to both increase (*e.g.* weight loss) and decrease (*e.g.* glucose ingestion) bone resorption do not result in reciprocal changes in OPG (Chailurkit *et al.*, 2008; Holecki *et al.*, 2008). As local expression of OPG mRNA levels in iliac bone biopsies correlates with fracture susceptibility (Abdallah *et al.*, 2005), taken together with the studies described above, findings from the current studies further question the strength of the relationship between circulating OPG and bone resorption.

Even if a sufficiently sensitive assay for RANKL should become available, to what extent circulating RANKL and OPG reflect their biological activity in the bone micro-environment remains a key question in understanding their relationship with bone turnover. In the current studies, the wide variation in both basal concentrations, and in its response to exercise, might reflect the contribution of tissues in addition to bone to its circulating concentrations. At the time that these studies were performed there was emerging evidence that OPG, in conjunction with RANKL and its receptor RANK, were the common link between bone, and the immune and cardiovascular systems (Doherty *et al.*, 2004). This was consistent with the idea that chronic diseases such arthrosclerosis (Libby, 2002) and diabetes (Dandona *et al.*, 2004) were possibly driven by inflammatory mechanisms, with the age-related increase in OPG being an adaptive response to the onset of a low-grade systemic inflammation (Ballou *et al.*, 1996; Bruunsgaard *et al.*, 1999; Dobbs *et al.*, 1999).

As OPG levels are elevated in inflammatory bowel conditions (Franchimont *et al.*, 2004; Moschen *et al.*, 2005) and familial Mediterranean fever (Yuksel *et al.*, 2009), and endurance running exerts strong effects on the immune system (Nieman, 1996), the increase in OPG with exercise might, in part, reflect an acceleration of inflammatory processes and tissue repair. Indeed, the 246 km run that was associated with a marked and sustained increase in OPG (Kerschan-Schindl *et al.*, 2009) also results in severe inflammation and evidence of cell repair (Papassotiriou *et al.*, 2008; Goussetis *et al.*, 2009), while OPG is elevated at 6 h and 2 days after a bout of severe muscle-damaging exercise (Philippou *et*

al., 2009). Changes in OPG as a marker of inflammation and/or tissue repair might explain why a prolonged increase was only seen in Study I where exercise was considerably more demanding than the other exercise protocols. That said, despite the demanding nature of the exercise there was still considerable variation in the OPG response with some subjects showing no change in OPG concentrations.

Inflammation also appears to underlie the pathology of insulin resistance and diabetes (Dandona *et al.*, 2004) and OPG is found in the walls of the vasculature in concentrations similar to that in bone (Olesen *et al.*, 2005) and elevated in the serum of patients with diabetes (Knudsen *et al.*, 2003). As exercise induces marked changes in glucose metabolism, changes in OPG with acute exercise might reflect changes in either glucose or insulin levels. Recently Knudsen *et al.* (2007) observed decreases in OPG concentrations during a hyperglycaemic (15 $mmol \cdot L^{-1}$) clamp which correlated with the increase in insulin. This finding was corroborated recently by Jørgensen *et al.* (2009) using an insulin infusion and is consistent with the dose-dependent decrease in OPG production from arterial wall tissue of diabetic patients in the presence of increasing insulin concentrations (Olesen *et al.*, 2005). If insulin negatively regulates OPG production in vascular tissue, it is possible that a decrease in insulin might result in an increase in OPG, although there is currently no data to support this idea. The two studies that report increased OPG with long-duration running (Ziegler *et al.*, 2005; Kerschan-Schindl *et al.*, 2009) did not measure insulin, although insulin levels are significantly reduced after a marathon (Utter *et al.*, 2002) and after only 28 km of an ultraendurance run (Noakes *et al.*, 1988). After 60 min of running in Study I, insulin was significantly decreased and OPG increased in RA subjects suggesting a possible link, while an insulin-dependent mechanism might also explain the tendency for the OPG response to be more marked and prolonged with more strenuous exercise, as seen in Studies I and III (Figure 9.4). That said, in ET subjects in Study I, OPG levels were increased 25% after only 20 min of exercise despite no change in insulin suggesting that factors other than changes in insulin are also involved.

Future studies that measure the time course of changes in bone metabolism, inflammatory markers and insulin are required to better understand the contribution of different systems to changes in OPG during exercise.

9.3 The effects of acute running on PTH and its relationship with changes in bone turnover markers

9.3.1 The effects of acute running on PTH concentrations

The studies described in this thesis have demonstrated that acute running is a potent stimulator of PTH concentrations. When examined together, and combined with the data from recent, well-controlled, laboratory studies (Guillemant *et al.*, 2004; Maïmoun *et al.*, 2006), a more complete description of the time course of this response is possible (Figure 9.5).

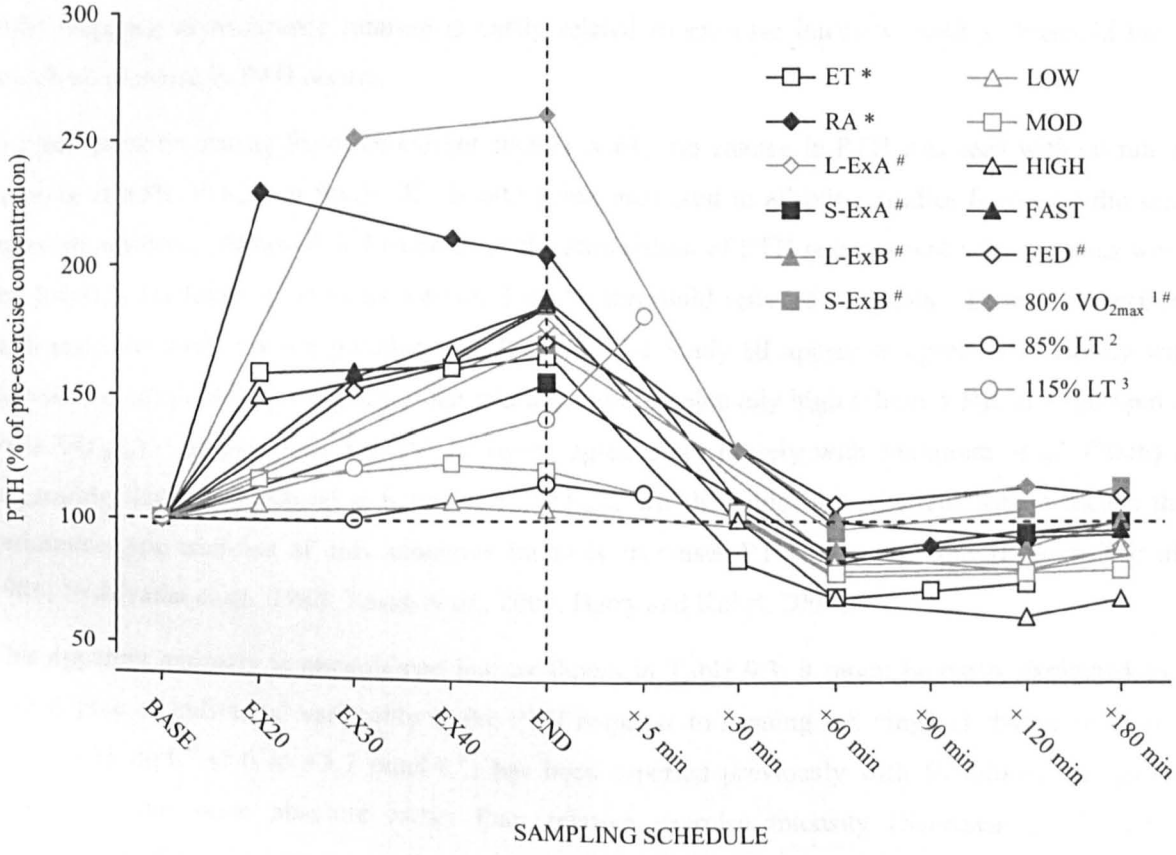

Figure 9.5. The percentage change (compared with a sample taken immediately before exercise), in PTH after 20, 30 and 40 min of exercise (EX20 to EX40), immediately after exercise (END) and from 15 min to 180 min after exercise (+15min to +180 min) lasting 50 or 60 min. * Data from first 60 min of exercise only. [1] 60 min (Guillemant *et al.*, 2004); [2] 50 min (Maïmoun *et al.*, 2006). # Subjects fed prior to exercise.

Studies I, III and IV demonstrate that PTH concentrations are increased rapidly (within 20-30 min) with the onset of acute, steady-state endurance running, while the magnitude of the increase exceeded the CV of the assay suggesting that it is not attributable to analytical variation. The increase in concentrations is maintained throughout 60 min of exercise and also when exercise is prolonged, intermittent and exhaustive. Importantly, when discussing the change in PTH with exercise in relation to its potential effects on bone, the current studies have showed for the first time that the PTH response to acute, steady-state endurance running is transient, with concentrations returning to

baseline within 30 min of the termination of exercise (Figure 9.5). Although these studies may have failed to detect that PTH remains increased up to 15 min post-exercise (Maïmoun *et al.*, 2006), this does not detract from the evidence that the PTH response is transient in nature.

In the young, healthy and physically-active population examined in the current studies, the PTH response to running was unaffected by improved training status, a bout of running performed 3 h previously, or the ingestion of a mixed meal prior to exercise. However, consistent with indirect evidence from incremental running (Salvesen *et al.*, 1994; Bouassida *et al.*, 2003) and direct evidence from steady-state cycling (Maïmoun *et al.*, 2006), Study III demonstrates for the first time that the PTH response to endurance running is partly related to exercise intensity, with a threshold below which no increase in PTH occurs.

A clear question arising from the current studies is why no change in PTH was seen with 60 min of running at 65% VO_{2max} in Study III, despite being increased in all other studies following the same exercise protocol. Although a threshold for the stimulation of PTH is consistent with previous work, the location (in terms of exercise intensity) of the threshold remains uncertain. Direct comparisons with previous work are not possible, but the results of Study III appear to agree more closely with Salvesen *et al.* (1994) in suggesting that this threshold is relatively high (above a HR of ~160 bpm or 65% VO_{2max}). Studies I, II and IV, however, agree more closely with Maïmoun *et al.* (2006) in suggesting that the threshold is below 65% VO_{2max}, which would be consistent with evidence that endurance-type exercise of only moderate intensity increases PTH concentrations (Ljunghall *et al.*, 1986; Nishiyama *et al.*, 1988; Tosun *et al.*, 2006; Barry and Kohrt, 2007).

This apparent anomaly is unexplained but, as shown in Table 9.3, it might be partly explained by a high degree of individual variability in the PTH response to running. A range of change in PTH of -15 to +35 $ng \cdot L^{-1}$ (1.6 to +3.7 $pmol \cdot L^{-1}$) has been reported previously with 50 min of incremental running at the same absolute rather than relative exercise intensity (Salvesen *et al.*, 1994). The findings from the current studies suggest that, even when relative exercise intensity is tightly controlled, a high degree of individual variability persists. Given the magnitude of the changes in PTH concentrations, analytical variability seems unlikely to account for this variability. In Study III, despite no significant increase in mean PTH concentrations in LOW and MOD, there were increases in PTH of between 15% and 80% in several individual subjects. As can be seen in Table 9.3, increases of this magnitude are well within the range of changes in PTH seen in Studies I, II and IV where significant increases in mean PTH concentrations were observed. As the results of Study III also show increased PTH concentrations in a greater number of subjects with increasing exercise intensity, the lack of significant increase in PTH at 65% VO_{2max} might be a statistical anomaly related to the variability in individual responses in a small group of subjects.

Table 9.3. Range of individual changes in PTH concentrations from pre-exercise to immediately post-exercise in Studies I-IV.

Study	Data Set	PTH (Range of percentage change from pre-exercise to immediately post-exercise)
I	RA	+18 to +215
I	ET	-7 to +169
II	L-ExA	-4 to +185
II	L-ExB	+15 to +109
II	S-ExA	+5 to +317
II	S-ExB	+17 to +119
III	LOW	-21 to +69
III	MOD	-24 to +77
III	HIGH	+12 to +171
IV	FAST	+3 to +219
IV	FED	-7 to +144

9.3.2 Are PTH concentrations reduced following acute, endurance running?

As can be seen from Figure 9.4 and Table 9.4, in addition to rapidly returning to baseline with the termination of exercise, in the majority of data sets, PTH concentrations are also significantly reduced from baseline in the first few hours post exercise. This might suggest that acute, endurance running results in a suppression of post-exercise PTH concentrations although, as can also be seen in Table 9.4, this is not a consistent finding.

As with increases during exercise, the magnitude of the decreases in PTH concentrations following exercise were markedly greater than the CV of the assay. Lower post-exercise concentrations could, however, simply be a reflection of the PTH circadian rhythm that reduces basal concentrations in the late morning. This might explain why the apparent reduction was not evident in Study IV, where exercise began at 1030, as basal concentrations would have already reached their daytime nadir during the recovery period. However, only a 5% decrease in PTH would be expected from the late morning to early afternoon due to the circadian rhythm (Joseph *et al.*, 2008) and the magnitude of the decrease in PTH concentration (up to 37%) following morning exercise far exceeds this.

Alternatively, exercise intensity or the overall 'metabolic load' of exercise (or both) might influence post-exercise PTH concentrations, as the strongest statistical differences were seen in Study I, while in Study III, the largest percentage decrease and the strongest statistical differences were seen in the HIGH condition (Table 9.4). Interestingly, the post-exercise decrease in PTH might be related in some way to exercise itself rather than to the increase in PTH during exercise, as PTH was reduced by 20% following the MOD condition in Study III, despite no increase in PTH during exercise.

Table 9.4. Mean percentage of pre-exercise PTH concentrations in the first 3 h post-exercise. Change calculated from a sample taken at 0800 h immediately prior to exercise unless otherwise stated.

Study	Data Set	+30 min	+60 min	+ 90 min	+120 min	+180 min
I	RA[1]	105	83 ***	90***	93 ***	-
I	ET[1]	84	70 ***	73***	76 ***	-
II	L-ExB[2]	-	84 *	-	81	90
II	S-ExB[2]	-	86 *	-	96	100
III	LOW	101	82	-	84	89
III	MOD	103	79 *	-	80 *	81 *
III	HIGH	100	70 ***	-	63 ***	70 ***
IV	FAST[3]	-	102	-	96	97
IV	FED[3]	-	106	-	114	110

[1] From statistical analysis performed on pooled data from RA and *et* group. [2] From statistical analysis performed on pooled data from L-ExA and S-ExB with change expressed as a percentage of value immediately pre-exercise (1415 h on Day 5). [3] From statistical analysis performed on pooled data from FAST and FED. As there was no significant change in PTH from 0800 h to 1015 h (pre-exercise), change is expressed as a percentage of value at 1015 h. * different ($P < 0.05$) from pre-exercise; *** different ($P < 0.001$) from pre-exercise.

An examination of the first half of Day 5 in Study II appears to support the idea that acute running reduces post-exercise PTH concentrations. During this period a single bout of running was performed in the SHORT condition while subjects were rested in the LONG condition. In the period following exercise in SHORT (1230 h to 1415 h), PTH concentrations were only 76-87% of 0800 h values while in LONG concentrations ranged from 94-108% of 0800 h values over the same period (Figure 9.6). When considered with the findings from Studies I and III, this further suggests that PTH concentrations could be reduced following a bout of running, particularly if the exercise is strenuous. Ultimately, a well-controlled study including a rested control group is required to confirm the presence of a decrease in post-exercise PTH concentration. The possible significance of such a reduction in PTH concentrations following exercise is discussed in the following section.

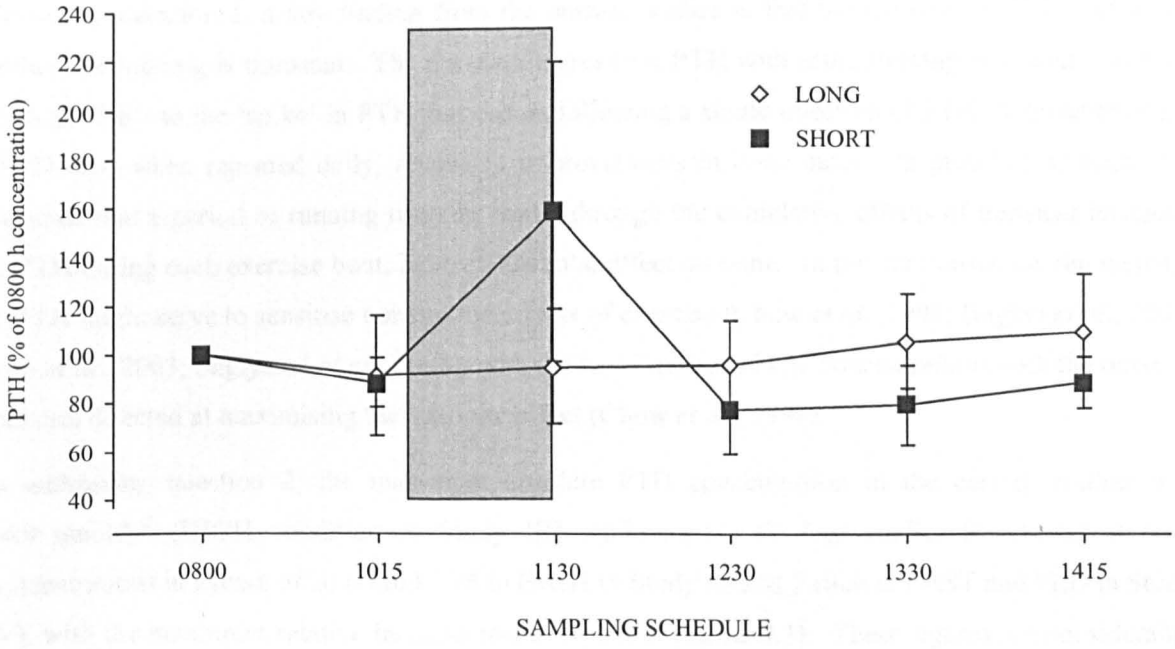

Figure 9.6. Percentage change in PTH concentrations from 0800 h to 1415 h on Day 5 in the LONG (open diamonds) and SHORT (filled squares) conditions in Study II. Grey box indicates 60 min of running at 65% VO_{2max} in SHORT only.

9.3.3 The relationship between changes in PTH and changes in bone turnover markers with acute running

In investigating the PTH response to acute exercise, a key question is whether that response, or repetitions of that response – such as in a period of training – might result in significant changes to bone itself. As PTH concentrations are increased during acute running, changes to bone might occur from repeated exposure to increased concentrations, a long-term change in the basal environment as a result of these increases, or a combination of the two. By measuring both PTH and specific BTM, the studies described in this thesis provide novel information about how changes in PTH with acute running might play a role in longer-term changes in bone turnover, and thus structure. In attempting to interpret if (and how) changes in PTH with acute running might affect bone turnover, the following questions must be considered:

1. How closely does the profile of the change in PTH with exercise resemble changes in PTH that are known to induce significant changes in BTM and bone structure?
2. If there is resemblance in the profile of the change in PTH, is there also resemblance in the magnitude of this change?
3. Finally, if there is evidence of a resemblance in both the profile and magnitude of change in PTH, are there also changes in BTM consistent with an effect of PTH?

Regarding question 1, a key finding from the current studies is that the increase in PTH with acute endurance running is transient. The transient increase in PTH with acute running is similar – at least in its profile – to the 'spike' in PTH that occurs following a single injection of PTH (Schwietert *et al.*, 1997) that, when repeated daily, results in improvements in bone mass. In principle at least, this suggests that a period of running training could, through the cumulative effects of transient increases in PTH during each exercise bout, have an anabolic effect on bone. In this circumstance, the increase in PTH might serve to sensitise bone to the effects of exercise (Chow *et al.*, 1998; Hagino *et al.*, 2001; Kim *et al.*, 2003; Sugiyama *et al.*, 2008), with the rapid increase in its concentrations with the onset of exercise directed at maximising the anabolic effect (Chow *et al.*, 1998).

In addressing question 2, the maximum absolute PTH concentration in the current studies was 14.9 $pmol \cdot L^{-1}$ (HIGH condition in Study III) while across all four studies 8 subjects showed concentrations in excess of 10 $pmol \cdot L^{-1}$ (4 in HIGH in Study III and 2 each in FAST and FED in Study IV), with the maximum relative increase just over 3-fold (Table 9.3). These figures are considerably lower than those reported following injections of PTH 1-84 and 1-34, where mean peak concentrations are 79 and 39 $pmol \cdot L^{-1}$ respectively (Schwietert *et al.*, 1997), and with relative increases, on average, of approximately 10-fold (Lindsay *et al.*, 1993). Alone, this might suggest that the magnitude of the increase in PTH with running might not be sufficient to elicit an anabolic effect in bone.

That said, rather than the maximum concentration, the primary determinate of whether PTH has anabolic effects appears to be the length of time concentrations remain above baseline levels (Frolik *et al.*, 2003). Although endogenous increases in PTH will almost certainly be of a lesser magnitude that those achieved with exogenous provision, in rats, EDTA-induced increases in PTH (3- to 6-fold) produce area under the curve values similar to those seen with a 5 $\mu g \cdot kg^{-1}$ injection of PTH, the latter of which results in bone formation (Fox *et al.*, 1997). Only very recently, however, have endogenous increases in PTH, achieved via a short-acting calcium-sensing receptor antagonist, been shown to increase bone strength in animals (Kumar *et al.*, 2010). In humans, these antagonists produce transient (several hours) increases (3- to 5.5-fold) in PTH that are consistent with those observed following PTH 1-34 treatment that resulted in bone formation (Kumar *et al.*, 2010). Thus, as with Question 1, at least in principle, these findings suggest that the duration and magnitude of increases in PTH observed during acute running might be capable of producing anabolic effects.

Regarding question 3, although the pattern and magnitude of changes in PTH with running might be consistent with a long-term anabolic effect on bone, there was no clear evidence of an increase in bone formation markers with one (Studies III and IV) or two (Study II) transient increases, or a single more prolonged, although still transient, increase (Study I). Where bone ALP levels were increased at 3 days and 4 days post exercise in Study III, this was a general effect of exercise rather than associated to the increase in PTH which occurred in the HIGH condition only. Bone formation markers are elevated after only 3 days of intermittent PTH therapy (Glover *et al.*, 2009). However, it is likely that

PTH therapy has a more potent anabolic effect and a greater number of bouts of exercise that induce changes in PTH, such as in a longer period of training, might be required before changes in bone formation markers are observed (Woitge *et al.*, 1998a).

Importantly, however, as discussed in Section 9.1, any interpretation of these studies is limited by the duration of the follow up period. Studies I and IV were associated with increases in β-CTX, albeit of considerably different duration. The temporal association (lagged by 30-60 min) between the increase in PTH and changes in β-CTX in Study IV is consistent with that seen with an EDTA infusion and a single injection of PTH (Zikán and Stepan, 2008), suggesting that the increase in β-CTX might be a result of the increase in PTH. As transient, exogenous and endogenous increases in PTH can have anabolic effects in bone, the increase in β-CTX with acute running in Study IV could be the early phase of a longer-term, PTH-mediated anabolic response.

In contrast, the increase in β-CTX from 1 to 4 days post-exercise in Study I might be less likely to reflect this type of response as, after 3 days of intermittent PTH therapy, β-CTX concentrations are significantly reduced (Glover *et al.*, 2009). Together with the results of Zikan and Stepan, (2008), this might suggest that the anabolic effects of PTH are preceded by only a transient increase in bone resorption. If, as suggested by the data from Study I, the increase in PTH was sustained for the full duration of FD and IEE, it is possible that this more prolonged increase was in excess of that optimal for bone formation (Dobnig and Turner, 1997; Frolik *et al.*, 2003) and instead induced a catabolic effect and a sustained increase in bone resorption.

Although these studies have not demonstrated that exercise duration is a factor in the PTH response to exercise, the rapid and sustained increase in PTH suggests that the duration of exposure to increased PTH concentrations might be determined by the duration of exercise. Indeed, as PTH is increased at the end of a long (2 h) bout of cycling (Barry and Kohrt, 2007) performed at an intensity that might not be expected to have any effect (Maïmoun *et al.*, 2006), this also suggests that exercise duration could be a factor in the PTH response. This might explain why cyclists appear to be particularly susceptible to negative changes in BMD (Barry and Kohrt, 2008) as, in addition to performing a non weight-bearing form of exercise, their training sessions tend to be much longer than those of runners, which might result in repeated, prolonged exposure to increased PTH concentrations. Well-controlled studies, including measurements of PTH and specific BTM, are needed to properly investigate the effects of different exercise durations.

Brahm *et al.* (1997a) have previously suggested that the most favourable effects of PTH on the skeleton would be achieved with low basal levels and large increases during exercise. It has also been proposed that the reduction in basal PTH levels following a physical training programme serves to exaggerate the transient increase during exercise and produce a stronger osteogenic effect (Vainionpää *et al.*, 2009). In a similar manner, it is possible that the apparent reduction in

post-exercise PTH concentrations in the current studies serves to accentuate the transient nature of the increase in PTH that precedes it, by maximising the relative difference in concentrations. With shorter (1 h) duration exercise, this might enhance the anabolic effect of each increase in PTH, whereas with a prolonged increase in PTH, such as that in Study I, the decrease might serve to limit the extent of PTH-mediated bone resorption once exercise ceases.

9.3.4 Mechanisms by which PTH concentrations might be increased during acute running

If, as suggested by Study IV, and more indirectly by Study I, the increase in PTH during moderate to high intensity endurance running results in increases in β-CTX, a specific marker of bone resorption, it is of interest to understand the mechanism(s) by which PTH is increased. In doing so, this might assist in the design of interventions which promote a more favourable response to acute exercise although, as discussed above, the exact nature of the PTH response which might promote a favourable change in BMD remains to be completely defined.

Although a reduction in blood pH and the resulting metabolic acidosis might contribute to the increase in PTH concentrations during maximal or supramaximal exercise, looking specifically at the current studies, increases in PTH during exercise despite only modest increases in blood lactate suggest metabolic acidosis is unlikely to play any role. In contrast, as epinephrine concentrations increase with exercise at only 40% VO_{2max} (Galbo *et al.*, 1975) the current studies are not able to rule out a contribution of changes in catecholamines in the increase in PTH. For example, as the increase in catecholamines is related to exercise intensity (Galbo *et al.*, 1975), changes in catecholamines might have contributed to the more consistent increase in PTH with increasing exercise intensity observed in Study III (Table 9.3). Further studies are required in humans that replicate the work of Body *et al.* (1983) to understand the effects of subtle changes in catecholamines on PTH, followed by studies of acute exercise that examine the time course of changes in both.

Under normal conditions, the serum iCa concentration is the key regulator of PTH secretion and in most circumstances, total Ca concentrations are considered a reliable indicator of iCa. Although exercise may result in a hemoconcentration, falsely elevating total Ca levels, expressing Ca levels 'adjusted' for albumin concentrations (*e.g.* ACa), as reported in the current studies, should account for this effect. As blood pH was also unlikely to be affected during running, this further suggests that changes in ACa in the current studies are likely a reasonable reflection of iCa concentrations. A similar change in total Ca and iCa would be consistent with the majority of studies that have examined changes in both in response to acute exercise (Aloia *et al.*, 1985; Ljunghall *et al.*, 1985; Nishiyama *et al.*, 1988; Henderson *et al.*, 1989; Rudberg *et al.*, 2000). In the current studies, PTH concentrations were increased when ACa concentrations were either increased (Studies I, II and III) or unchanged (Study IV) suggesting, albeit indirectly, that a decrease in iCa is unlikely to account for the increases.

The numerous reports in the literature of increased total Ca concentrations during endurance exercise might be explained by the rapid increase in PTH concentrations, with PTH stimulating calcium reabsorption in the kidney and the liberation of calcium from bone by osteoclastic resorption. Evidence from the current studies, however, of an increase in ACa but unchanged PTH (LOW and MOD in Study III) and no change in ACa despite increased PTH (Study IV) does not support this idea.

An interesting observation from the current studies is that the more pronounced decreases in post-exercise PTH concentrations (Studies I and III) occurred when post-exercise ACa concentrations remained elevated. In Study I, the tendency for a greater increase in post-exercise ACa concentrations in *et* compared with RA (+11% *vs* +7%) was accompanied by a tendency for a greater reduction in post-exercise PTH (-24 to 30% *vs* -10 to 17%), while in Study III, the tendency for a greater reduction post-exercise PTH concentrations in HIGH compared with MOD and LOW (-30 to 36% *vs* -11 to 21%) was accompanied by a tendency for higher post-exercise ACa concentrations (+6% *vs* +3%). Therefore, it is possible that although PTH and ACa do not appear to regulate each other during exercise, with the termination of exercise the normal relationship between Ca and PTH is restored.

There is evidence in animals that subtle and short-term increases in PO_4 can stimulate PTH concentrations (Martin *et al.*, 2005), while in humans, increases in PO_4 precede and correlate with subsequent changes in PTH (Ahmad *et al.*, 2003). However, the results of Study III provide direct evidence to argue against PO_4 as the mediator of the increase in PTH during acute, endurance running, corroborating findings from a recent study of cycling (Maïmoun *et al.*, 2006). The lack of significant relationships between changes in PO_4 and PTH during running in the current studies and the high degree of variability in individual PTH responses compared to the relative regularity of increases in PO_4 also, albeit more indirectly, support this argument. That said, the lack of any measurements taken in the first 20 min of running in any study, provides a rationale for future studies to examine the acute time course of this relationship in more detail. As with catecholamines, further studies in resting humans are also required to understand the effects of subtle changes in PO_4 on PTH concentrations.

9.4 Limitations

There are a number of factors that should be considered when interpreting the results of these studies. The first is possible influence of the life-long exercise history of the subjects studied. As mentioned previously, the responsiveness of bone to mechanical loading quickly diminishes as the duration of a programme of loading is extended (Turner *et al.*, 1992; Kim *et al.*, 2003; Saxon *et al.*, 2005; Schriefer *et al.*, 2005). In order to ensure that they were able to complete the exercise protocols, particularly the more strenuous ones used in Studies I, II and III, subjects were selected based on their history of weight-bearing and familiarity with running. The implication of the lack of 'novelty' of the exercise stimulus specifically in the comparison on the RA and *et* groups in Study I has already been

discussed, but it might also be a factor in the remaining three studies, diminishing the general response of bone to acute running. As it is the bone formation response that diminishes with continued loading, this might explain the lack of any sustained effect of any of the exercise protocols on bone formation markers up to 4 days post-exercise. Equally, a history of weight-bearing exercise in these subjects might have resulted in an osteogenic response that increased their skeletal resistance to deformation (Turner and Burr, 1993) by the types of forces induced by treadmill running. Although it is not yet possible to confirm if a single bout of endurance exercise induces microdamage in human bone, this increased resistance might result in less damage and associated remodelling with the exercise protocols used in this thesis and, as with bone formation markers, more marked changes in bone resorption markers might be observed with truly naive subjects.

Secondly, as highlighted in Chapter II, a limitation of all studies of the effects of acute exercise on bone metabolism in humans is that it is not possible to differentiate 'pure' mechanical effects from those resulting from metabolic and/or endocrine changes. This is of particular importance when the exercise protocol utilised results in a marked difference in mechanical loads between individuals (such as in Study I) or in a difference between experimental conditions as an unavoidable consequence of a deliberate manipulation of a different variable such as cardiovascular exercise intensity (as in Study III). However, even with a within-subject study design and the tight standardisation of exercise duration and cardiovascular exercise intensity (as in Study IV), there remains a complex situation regarding individual mechanical loading characteristics. As discussed in Chapter IV in relation to the findings from Study I, running speed, body mass, and the degree of skeletal adaptation to the prevailing mechanical environment, the latter of which will be related to both body mass (Reid *et al.*, 2006) and habitual physical activity (Turner and Burr, 1993), will all influence the number of strains, as well strain magnitude and strain rate during a 'standardised' bout of acute, endurance running. Attempting to standardise mechanical loading during acute running, therefore, requires consideration of both the number and magnitude of loads and would result in a markedly different type of study that would likely result in considerable variation between individuals in terms of exercise duration and cardiovascular exercise intensity.

The third and final limitation relates to the statistical power of these studies. As the study designs were novel, is was not possible to perform *a priori* power analysis to determine the sample size needed for each study. Given the relatively low number of subjects and the high level of variability in some of the markers examined, it is possible the sample size might have been inadequate to detect genuine statistical differences. This issue might be of particular importance in relation to the statistical analysis approach taken in this thesis. The statistical methods used were thorough, testing data sets firstly for a significant main effects of *Time* and a significant *Group/Condition* x *Time* interaction, and then using *post-hoc* tests to test for significant differences between time points within a group/condition and also for differences between groups/conditions at individual time points.

With small subject numbers and a high level of marker variability, statistical tests might have insufficient power to detect real differences between data sets should they exist. Indeed there are several findings in this thesis that might be a result of inadequate statistical power, including the lack of a significant *Condition* x *Time* interaction for OPG in Study I and for P1NP in Study III.

In contrast to this thesis, previous studies that have analysed multiple groups, either including a non-exercising control group or comparing different exercise conditions, have not used such rigorous methods. For example, Wallace *et al.*, (2000) compared changes in bone markers with exercise (n = 8) to a non-exercising control group (n = 8) and tested data sets using a general linear model, and report a number of significant *Group* x *Time* interactions. However, they did not perform any post-hoc tests in an attempt to identify the location (*i.e.* at which time points) at which differences between bone marker concentrations in the two groups existed. Ehrnborg *et al.*, (2003) compared changes in bone turnover markers with exercise between men and women. However, the authors did not test their data for a significant *Group* x *Time* interaction but instead used only the Wilcoxon signed rank test to test for significant differences across time within each group. Similarly, both Maïmoun *et al.*, (2006) and Herrmann *et al.*, (2007) compared changes in bone markers with exercise at different cardiovascular exercise intensities in small (n = 7-9) groups of subjects but did not test their data for a significant *Group* x *Time* interaction. Like Ehrnborg *et al.*, (2003), these authors only examined their data for significant changes across time within each group. It is possible, therefore, that had these studies employed similar statistical analyses to those used in this thesis, they may have also failed to identity statistical differences between experimental groups.

In conclusion, given the possibility of a lack of adequate statistical power, it may be appropriate to consider the studies in this thesis exploratory and the data from them should be used to design future studies that are powered to allow the outcomes of similar statistical analyses methods to be accepted with a greater degree of confidence.

9.5 The role of increases in the circulating concentrations of the cytoines TNF-α, IL-1β and IL-6 in changes in bone turnover with acute running

Finally, this thesis also examined a novel hypothesis that increases in circulating concentrations of the cytokines TNF-α, IL-1β and IL-6 during acute running might, in part, be mediating changes in bone resorption. Thus, the current studies are the first to simultaneously measure these cytokines and markers of bone turnover in response to the same exercise protocols. The studies were not designed to determine cause and effect in this relationship, but to seek evidence that might warrant a more thorough investigation of such a mechanism. From these studies, due to unchanged concentrations during exercise, or only modest and transient increases, changes in IL-1β would appear least likely to contribute to changes in bone resorption, followed closely by TNF-α, which showed similar responses. The most appealing – albeit still indirect – evidence for the involvement of cytokines comes from

measurements of IL-6, with concentrations increased during all exercise protocols and enhanced further by more strenuous running. Based on changes in IL-6 and β-CTX, this would most likely occur through a marked increase in the production of IL-6 during, but not after, highly strenuous exercise as, despite increased β-CTX and evidence of muscle damage on the four follow-up days in Study I, there was no evidence of a prolonged increase in IL-6 concentrations.

Key to this mechanism is the ability of IL-6 that is released into the circulation during exercise to function in an endocrine manner, with osteoclasts as target cells. As osteoclasts do not appear to express IL-6R in physiological conditions (Tamura *et al.*, 1993), the presence of increased levels of sIL-6R with exercise would seem to be a necessary pre-requisite. With recent evidence that sIL-6R is indeed increased during exercise (Gray *et al.*, 2008; Gray *et al.*, 2009), potentially rendering osteoclasts target cells for circulating IL-6 (Tamura *et al.*, 1993), and a possible mediating role of increased PTH in the appearance of sIL-6R (Mitnick *et al.*, 2001), the role of circulating IL-6 in the bone resorption response to acute, endurance exercise seems to warrant a more thorough investigation. Interestingly, estrogen-deficient women have increased skeletal sensitivity to the resorbing action of PTH infusion (Cosman *et al.*, 1993) and the greater increase in bone resorption markers with estrogen-deficiency is accompanied by a greater increase in IL-6 and sIL-6R (Masiukiewicz *et al.*, 2002). If the increase in sIL-6R during exercise is also a result of PTH-mediated hepatic release (Mitnick *et al.*, 2001), it is an intriguing possibility that amenorrheic female runners might also have an enhanced sIL-6R response to increased PTH during exercise. Although there is no evidence of IL-6 production by the liver during exercise (Febbraio *et al.*, 2003), when combined with IL-6 released from working muscle, this might also result in an exaggerated increase in bone resorption. Such an effect might, in part, contribute to the reduced BMD reported in this population (Pettersson *et al.*, 1999).

Many of the insights into the possible endocrine functions of muscle-derived IL-6 have come from studies that have infused recombinant IL-6 into human subjects (Steensberg *et al.*, 2003; Febbraio *et al.*, 2004). As these studies have been searching for potential glucoregulatory and lipolytic effects of IL-6, they have focused mainly on muscle, adipose tissue and the liver. However, with novel evidence of the increase in sIL-6R with exercise, future studies that measure BTM during a physiologically-relevant infusion of IL-6 will provide useful information regarding its potential to mediate the changes in bone turnover observed with acute, endurance exercise. In addition, as IL-6 release from working muscle can be manipulated in a number of ways, including carbohydrate ingestion (Nehlsen-Cannarella *et al.*, 1997), lowering pre-exercise muscle glycogen (Keller *et al.*, 2001; Steensberg *et al.*, 2001) and vitamin C and E supplementation (Fischer *et al.*, 2004), these methods might also be used to examine the impact of varying systemic IL-6 concentrations on changes in sIL-6R and BTM during exercise itself.

9.6 Conclusion

These studies have shown that a single bout of endurance running results in an increase in β-CTX concentrations, which, when measured in fasted, early morning samples, can last for up to 4 days if exercise is strenuous and exhaustive. The β-CTX response to running was modestly affected by exercise intensity and of a greater magnitude and duration in pre-fed humans, while the timing of a 'baseline' blood sample and of exercise itself, as well as the nutritional status of subjects prior to exercise appear to affect both the direction and magnitude of the β-CTX response. Thus, the factors that determine the duration and magnitude of the increase in bone resorption with acute endurance running remain to be fully defined. Changes in the circulating concentrations of the pro-inflammatory cytokines TNF-α and IL-1β are unlikely to be involved in the β-CTX response to running. However, with marked and sustained increases, particularly with more strenuous exercise, a contributory effect of circulating IL-6 is possible although such a mechanism remains purely speculative and requires considerable further study.

Apart from transient increases during exercise, bone formation markers remained largely unchanged in response to acute, endurance running, at least up to 4 days post-exercise. This might indicate that acute endurance running, particularly if strenuous and exhaustive, can induce a transient imbalance in bone turnover, favouring bone resorption, although any conclusions regarding the net effects of running on bone turnover remain limited by the duration of the follow-up period.

Neither Cr-corrected pyridinolines nor pyridinoline 'output' measured from a fasted, early morning, second-void sample accurately reflect β-CTX concentrations. Both increases in Cr concentrations with exercise, and considerable individual variability in Cr concentrations and urine volume in a SMV, likely hamper the precision of each technique, and neither appears to be useful in terms of assessing bone resorption under these conditions.

Circulating OPG concentrations are increased during endurance running with the increase tending to be more pronounced and longer when the total 'metabolic load' of exercise is greatest. A portion of this increase likely reflects analytical variability, although the magnitude and rate of the increase was greater than could be attributable to analytical variability of the circadian rhythm, suggesting a genuine effect of exercise. However, across all studies, irrespective of the metabolic load of exercise, there is considerable variability in the magnitude of the individual OPG response and decreased concentrations in some subjects. Despite increases in mean OPG and β-CTX in some studies, OPG concentrations failed to closely reflect acute differences in β-CTX, suggesting that it is not an accurate indicator of bone resorption under these conditions.

A bout of endurance running results in a transient increase in PTH, with concentrations increased within 20 min of the onset of exercise, maintained throughout exercise and returning to baseline within 30 min of recovery. This increase is unaffected by improved training status, prior exercise and

pre-exercise nutritional status, but occurs only above a certain exercise intensity, although there is evidence, in the form of a wide variation in the PTH response, that this threshold may vary between individuals. Despite the transient increase in PTH being consistent with a profile that might be an anabolic stimulus for bone, at least up to four days post-exercise, there is no evidence of an association between increased PTH and increases in bone formation markers. In relation to changes in PTH concentrations, neither ACa nor PO_4 appear likely to mediate the increase in PTH during acute, endurance running.

There remains much to be learned regarding the metabolic response of bone to acute, exercise. However, by applying appropriate experimental control, these studies have both corroborated findings from previous studies of acute cycling and provided novel information with respect to changes in bone metabolism that occur with acute, endurance running. This information will serve as a basis for the design and interpretation of future investigations.

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Appendices

Appendix A

Details of the three individual menus in the experimental diets provided to subjects in Studies I to IV

Study I: RA Group

ID	MEASURE	MENU A	MENU B	MENU C	MEAN	FFM (kg)	g C/kg FFM
RA001	KCAL	2194	2197	2000	2130		
	CHO (g)	438	438	438	438	54.7	8.00
	CHO (%)	75	75	75	75		
	FAT (g)	30	26	25	27		
	FAT (%)	12	11	10	11		
	PROTEIN (g)	68	77	81	75		
	PROTEIN (%)	13	14	15	14		
	Ca (mg)	574	922	749	748		
RA002	KCAL	2523	2523	2521	2522		
	CHO (g)	499	498	498	498	62.3	7.99
	CHO (%)	75	74	75	75		
	FAT (g)	31	30	30	30		
	FAT (%)	11	11	11	11		
	PROTEIN (g)	86	97	89	91		
	PROTEIN (%)	14	15	14	14		
	Ca (mg)	883	1440	1183	1169		
RA003	KCAL	2869	2739	2740	2783		
	CHO (g)	510	510	509	509	63.7	8.00
	CHO (%)	71	70	70	70		
	FAT (g)	40	48	45	44		
	FAT (%)	13	16	15	15		
	PROTEIN (g)	104	99	103	102		
	PROTEIN (%)	16	14	15	15		
	Ca (mg)	1588	902	1094	1195		
RA004	KCAL	3188	3186	3189	3188		
	CHO (g)	506	506	507	507	63.3	8.00
	CHO (%)	58	60	58	59		
	FAT (g)	98	90	92	93		
	FAT (%)	27	25	26	26		
	PROTEIN (g)	119	119	130	123		
	PROTEIN (%)	15	15	16	15		
	Ca (mg)	1754	1786	1673	1738		
RA005	KCAL	2532	2531	2534	2532		
	CHO (g)	445	443	448	445	55.6	8.02
	CHO (%)	66	68	66	67		
	FAT (g)	50	45	49	48		
	FAT (%)	18	17	17	17		
	PROTEIN (g)	103	94	104	100		
	PROTEIN (%)	16	15	16	16		
	Ca (mg)	445	723	725	631		

ID	MEASURE	MENU A	MENU B	MENU C	MEAN	FFM (kg)	g C/kg FFM
RA006	KCAL	2301	2360	2432	2364		
	CHO (g)	461	460	462	461	57.7	7.99
	CHO (%)	75	74	71	73		
	FAT (g)	25	29	38	31		
	FAT (%)	10	11	14	12		
	PROTEIN (g)	86	87	91	88		
	PROTEIN (%)	15	15	15	15		
	Ca (mg)	1028	1154	695	959		
RA007	KCAL	2936	2937	2937	2937		
	CHO (g)	485	485	484	485	60.6	8.00
	CHO (%)	63	62	62	62		
	FAT (g)	74	74	79	76		
	FAT (%)	23	23	24	23		
	PROTEIN (g)	106	107	105	106		
	PROTEIN (%)	15	15	14	15		
	Ca (mg)	1067	1385	559	1004		
RA008	KCAL	3009	3009	3008	3009		
	CHO (g)	525	525	526	525	65.6	8.01
	CHO (%)	65	68	65	66		
	FAT (g)	62	54	62	59		
	FAT (%)	19	17	18	18		
	PROTEIN (g)	121	108	121	117		
	PROTEIN (%)	16	15	16	16		
	Ca (mg)	1227	1716	1045	1330		
RA009	KCAL	2969	2972	2968	2969		
	CHO (g)	512	511	511	511	64.0	7.99
	CHO (%)	66	64	65	65		
	FAT (g)	59	66	67	64		
	FAT (%)	18	20	20	19		
	PROTEIN (g)	119	117	113	116		
	PROTEIN (%)	16	16	15	16		
	Ca (mg)	1772	1065	1237	1356		
RA010	KCAL	2558	2555	2558	2557		
	CHO (g)	512	511	512	511	64.5	7.94
	CHO (%)	76	75	75	75		
	FAT (g)	26	26	27	27		
	FAT (%)	9	9	10	9		
	PROTEIN (g)	95	101	99	98		
	PROTEIN (%)	15	16	15	15		
	Ca (mg)	1454	1315	1016	1262		
RA011	KCAL	2660	2662	2675	2666		
	CHO (g)	425	424	425	425	53.3	7.97
	CHO (%)	60	60	60	60		
	FAT (g)	73	76	72	74		
	FAT (%)	25	26	24	25		
	PROTEIN (g)	103	97	108	102		
	PROTEIN (%)	15	15	16	15		
	Ca (mg)	1264	954	768	995		

Study I: ET Group

ID	MEASURE	MENU A	MENU B	MENU C	MEAN	FFM (kg)	g CHO/kg FFM
ET001	KCAL	2565	2564	2565	2565		
	CHO (g)	407	406	407	407	50.8	8.00
	CHO (%)	60	60	60	60		
	FAT (g)	69	74	74	72		
	FAT (%)	24	26	26	25		
	PROTEIN (g)	101	92	91	95		
	PROTEIN (%)	16	14	14	15		
	Ca (mg)	1632	906	1525	1353		
ET002	KCAL	3636	3630	3634	3633		
	CHO (g)	470	473	470	471	59.0	7.99
	CHO (%)	49	48	49	49		
	FAT (g)	137	152	149	146		
	FAT (%)	34	37	37	36		
	PROTEIN (g)	156	130	122	136		
	PROTEIN (%)	17	14	14	15		
	Ca (mg)	1489	678	1155	1108		
ET003	KCAL	2918	2921	2920	2920		
	CHO (g)	430	431	430	430	53.7	8.01
	CHO (%)	55	56	55	55		
	FAT (g)	96	96	92	95		
	FAT (%)	30	30	29	30		
	PROTEIN (g)	106	100	116	108		
	PROTEIN (%)	15	14	16	15		
	Ca (mg)	978	1686	1726	1464		
ET004	KCAL	2546	2551	2547	2548		
	CHO (g)	513	513	514	513	64.2	8.00
	CHO (%)	75	76	76	76		
	FAT (g)	27	21	20	23		
	FAT (%)	9	7	7	8		
	PROTEIN (g)	98	104	110	104		
	PROTEIN (%)	15	17	17	16		
	Ca (mg)	1294	1093	1083	1088		
ET005	KCAL	2746	2736	2735	2739		
	CHO (g)	440	438	437	438	54.8	8.00
	CHO (%)	60	60	60	60		
	FAT (g)	69	76	70	71		
	FAT (%)	23	25	23	24		
	PROTEIN (g)	119	103	117	113		
	PROTEIN (%)	17	15	17	16		
	Ca (mg)	563	1599	847	1003		
ET006	KCAL	2504	2407	2384	2432		
	CHO (g)	459	455	457	457	57.3	7.97
	CHO (%)	69	71	72	71		
	FAT (g)	46	40	39	42		
	FAT (%)	17	15	15	16		
	PROTEIN (g)	93	87	81	87		
	PROTEIN (%)	15	15	14	15		
	Ca (mg)	713	945	842	833		

ID	MEASURE	MENU A	MENU B	MENU C	MEAN	FFM (kg)	g C/kg FFM
ET007	KCAL	3122	3724	3205	3350		
	CHO (g)	433	431	431	432	53.7	8.04
	CHO (%)	53	43	51	49		
	FAT (g)	109	164	113	129		
	FAT (%)	32	40	32	35		
	PROTEIN (g)	121	159	134	138		
	PROTEIN (%)	16	17	17	17		
	Ca (mg)	1028	1571	1076	1225		
ET008	KCAL	2700	2736	2892	2776		
	CHO (g)	519	520	521	520	65.1	7.990
	CHO (%)	72	71	68	70		
	FAT (g)	40	54	64	53		
	FAT (%)	13	18	20	17		
	PROTEIN (g)	97	74	90	87		
	PROTEIN (%)	14	11	12	12		
	Ca (mg)	806	922	1212	980		
ET009	KCAL	2554	2552	2660	2588		
	CHO (g)	459	459	459	459	57.4	7.997
	CHO (%)	68	67	65	67		
	FAT (g)	57	55	56	56		
	FAT (%)	20	19	19	19		
	PROTEIN (g)	78	84	108	90		
	PROTEIN (%)	12	13	16	14		
	Ca (mg)	1452	1472	1404	1442		
ET010	KCAL	2648	2943	2867	2819		
	CHO (g)	439	436	439	438	55.8	7.85
	CHO (%)	60	56	57	58		
	FAT (g)	79	101	88	89		
	FAT (%)	26	31	28	28		
	PROTEIN (g)	99	99	109	102		
	PROTEIN (%)	14	14	15	14		
	Ca (mg)	960	1658	923	1180		

Study I: CON Group

ID	MEASURE	MENU A	MENU B	MENU C	MEAN	FFM (kg)	g C/kg FFM
CON001	KCAL	2608.79	2517.64	2599.26	2575.2		
	CHO (g)	497.89	498.52	498.38	498.3	62.3	7.99
	CHO (%)	72	74	72	72.7		
	FAT (g)	30.99	27.61	41.22	33.3		
	FAT (%)	11	10	14	11.7		
	PROTEIN (g)	108.21	100.6	89.3	99.4		
	PROTEIN (%)	17	16	14	15.7		
	Ca (mg)	851	597	1063	837		
CON002	KCAL	2375	2472	2400	2415.7		
	CHO (g)	451.65	452.58	451.88	452.0	56.6	7.99
	CHO (%)	71	69	71	70.3		
	FAT (g)	35.92	46.04	40.75	40.9		
	FAT (%)	14	17	15	15.3		
	PROTEIN (g)	89.67	90.65	85.31	88.5		
	PROTEIN (%)	15	15	14	14.7		
	Ca (mg)	1039	1407	933	1126		
CON003	KCAL	2787.5	3024.7	2846.6	2886.3		
	CHO (g)	497.04	496.8	496.57	496.8	62.2	7.99
	CHO (%)	67	62	65	64.7		
	FAT (g)	64.3	87.2	60.8	70.8		
	FAT (%)	21	26	19	22.0		
	PROTEIN (g)	86.4	95.2	110	97.2		
	PROTEIN (%)	12	13	15	13.3		
	Ca (mg)	1261	1454	1696	1470		
CON004	KCAL	2097.78	2087.64	2170.11	2118.5		
	CHO (g)	387.49	387.68	387.67	387.6	48.5	7.99
	CHO (%)	69	70	67	68.7		
	FAT (g)	33.26	39.98	49.11	40.8		
	FAT (%)	14	17	20	17.0		
	PROTEIN (g)	86.29	68.23	68.36	74.3		
	PROTEIN (%)	16	13	13	14.0		
	Ca (mg)	1259	1342	954	1185		
CON005	KCAL	2250.5	2244.74	2350.62	2282.0		
	CHO (g)	403.54	404.57	404.98	404.4	50.6	8.00
	CHO (%)	67	68	65	66.7		
	FAT (g)	44.66	51.22	52.26	49.4		
	FAT (%)	18	21	20	19.7		
	PROTEIN (g)	84.27	66.61	90.33	80.4		
	PROTEIN (%)	15	12	15	14.0		
	Ca (mg)	652	1032	1265	983		
CON006	KCAL	2453.05	2421.13	2361.15	2411.8		
	CHO (g)	415.08	415.95	414.61	415.2	51.9	8.00
	CHO (%)	64	65	66	65.0		
	FAT (g)	55.38	54.45	49.04	53.0		
	FAT (%)	21	20	19	20.0		
	PROTEIN (g)	90.09	88.47	91.14	89.9		
	PROTEIN (%)	15	15	15	15.0		
	Ca (mg)	1319	1022	1289	1210		

ID	MEASURE	MENU A	MENU B	MENU C	MEAN	FFM (kg)	g C/kg FFM
CON007	KCAL	2612.6	2513.5	2355.1	2493.7	67.2	8.10
	CHO (g)	545.2	546.7	540.3	544.1		
	CHO (%)	57	60	64	60.3		
	FAT (g)	75.6	71.5	53.8	67.0		
	FAT (%)	26	26	21	24.3		
	PROTEIN (g)	107	90.5	92.5	96.7		
	PROTEIN (%)	16	14	16	15.3		
	Ca (mg)	642	1687	976	1102		
CON008	KCAL	2558.4	2543.6	2583.8	2561.9	69.7	7.94
	CHO (g)	552.5	556.8	550	553.1		
	CHO (%)	61	61	61	61.0		
	FAT (g)	64.4	64.3	68.8	65.8		
	FAT (%)	23	23	24	23.3		
	PROTEIN (g)	102	104.1	99.8	102.0		
	PROTEIN (%)	16	16	15	15.7		
	Ca (mg)	1532	1342	1878	1584		
CON009	KCAL	2544.4	2584	2386.5	2505.0	64.7	7.90
	CHO (g)	510.4	510.3	513.2	511.3		
	CHO (%)	54	53	59	55.3		
	FAT (g)	94.7	95.7	64.4	84.9		
	FAT (%)	33	33	24	30.0		
	PROTEIN (g)	80.7	88.4	102.5	90.5		
	PROTEIN (%)	13	14	17	14.7		
	Ca (mg)	1450	1350	1523	1441		
CON010	KCAL	3303.3	3361.94	3343.3	3336.2	67.9	8.00
	CHO (g)	542.36	544.24	542.36	543.0		
	CHO (%)	62	61	61	61.3		
	FAT (g)	101.46	98.35	83.77	94.5		
	FAT (%)	28	26	23	25.7		
	PROTEIN (g)	89.98	108.8	138.95	112.6		
	PROTEIN (%)	11	13	17	13.7		
	Ca (mg)	856	953	997	935		

Study II

ID	MEASURE	MENU A	MENU B	MENU C	MEAN	FFM (kg)	g CHO/kg FFM
001	KCAL	2749	2720	2756	2742		
	CHO (g)	443	449	438	443	73.7	6.02
	CHO (%)	60	62	60	61		
	FAT (g)	75	70	75	73		
	FAT (%)	25	23	25	24		
	PROTEIN (g)	102	103	110	105		
	PROTEIN (%)	15	15	16	15		
	Ca (mg)	1425	1457	1211	1364		
002	KCAL	3164	3143	3151	3153		
	CHO (g)	377	380	380	379	62.8	6.04
	CHO (%)	45	46	45	45		
	FAT (g)	142	142	134	139		
	FAT (%)	40	41	38	40		
	PROTEIN (g)	119	107	129	118		
	PROTEIN (%)	15	14	16	15		
	Ca (mg)	1112	1403	820	1112		
003	KCAL	3228	3407	3346	3327		
	CHO (g)	392	395	395	394	66.4	5.94
	CHO (%)	53	52	54	53		
	FAT (g)	108	122	112	114		
	FAT (%)	30	32	30	31		
	PROTEIN (g)	141	138	134	137		
	PROTEIN (%)	17	16	16	16		
	Ca (mg)	1612	1783	1562	1652		
004	KCAL	2552	2545	2587	2561		
	CHO (g)	416	419	419	418	69.7	6.00
	CHO (%)	61	62	61	61		
	FAT (g)	62	67	66	65		
	FAT (%)	22	24	23	23		
	PROTEIN (g)	111	94	106	104		
	PROTEIN (%)	17	15	16	16		
	Ca (mg)	1066	1131	1359	1185		
005	KCAL	2737	2740	2741	2739		
	CHO (g)	388	389	389	389	64.7	6.01
	CHO (%)	53	53	53	53		
	FAT (g)	100	98	91	96		
	FAT (%)	33	32	30	32		
	PROTEIN (g)	98	102	115	105		
	PROTEIN (%)	14	15	17	15		
	Ca (mg)	1148	898	938	995		
006	KCAL	2890	2593	2878	2787		
	CHO (g)	452	388	456	432	75.4	5.73
	CHO (%)	59	56	59	58		
	FAT (g)	68	76	86	77		
	FAT (%)	21	26	27	25		
	PROTEIN (g)	148	116	101	122		
	PROTEIN (%)	20	18	14	17		
	Ca (mg)	2000	1511	2042	1851		

ID	MEASURE	MENU A	MENU B	MENU C	MEAN	FFM (kg)	g CHO/kg FFM
007	KCAL	2214	2225	2202	2214		
	CHO (g)	365	364	364	364	60.3	6.04
	CHO (%)	62	61	65	63		
	FAT (g)	53	61	48	54		
	FAT (%)	20	24	20	21		
	PROTEIN (g)	93	80	80	84		
	PROTEIN (%)	17	14	15	15		
	Ca (mg)	793	1091	1030	971		
008	KCAL	2613	2514	2355	2494		
	CHO (g)	399	403	400	400	67.2	5.96
	CHO (%)	57	60	64	60		
	FAT (g)	76	72	54	67		
	FAT (%)	26	26	21	24		
	PROTEIN (g)	107	91	93	97		
	PROTEIN (%)	16	14	16	15		
	Ca (mg)	1649	797	949	1132		
009	KCAL	2558	2544	2584	2562		
	CHO (g)	419	415	417	417	69.7	5.98
	CHO (%)	61	61	61	61		
	FAT (g)	64	64	69	66		
	FAT (%)	23	23	24	23		
	PROTEIN (g)	102	104	100	102		
	PROTEIN (%)	16	16	15	16		
	Ca (mg)	1177	1660	1289	1375		
010	KCAL	2544	2584	2387	2505		
	CHO (g)	366	366	373	368	61.7	5.97
	CHO (%)	54	53	59	55		
	FAT (g)	95	96	64	85		
	FAT (%)	33	33	24	30		
	PROTEIN (g)	81	88	103	91		
	PROTEIN (%)	13	14	17	15		
	Ca (mg)	1535	1087	1376	1333		

Study III

ID	MEASURE	MENU A	MENU B	MENU C	MEAN	FFM (kg)	g CHO/kg FFM
001	KCAL	2461	2345	2349	2385		
	CHO (g)	354	328	337	340	71.6	4.74
	CHO (%)	54	52	54	53		
	FAT (g)	79	94	79	84		
	FAT (%)	29	32	30	30		
	PROTEIN (g)	17	16	16	16		
	PROTEIN (%)	106	82	92	94		
	Ca (mg)	1269	1255	1240	1255		
002	KCAL	2050	2073	2123	2082		
	CHO (g)	298	319	312	310	64.3	4.81
	CHO (%)	54	58	55	56		
	FAT (g)	77	63	67	69		
	FAT (%)	30	27	28	28		
	PROTEIN (g)	83	78	88	83		
	PROTEIN (%)	16	12	17	15		
	Ca (mg)	793	774	743	770		
003	KCAL	2614	2622	2632	2623		
	CHO (g)	389	402	397	396	68.4	5.79
	CHO (%)	56	57	56	56		
	FAT (g)	77	72	84	78		
	FAT (%)	27	25	29	27		
	PROTEIN (g)	115	118	98	110		
	PROTEIN (%)	18	18	15	17		
	Ca (mg)	1227	1266	1277	1257		
004	KCAL	3149	2963	3057	3056		
	CHO (g)	453	429	419	434	63.7	6.81
	CHO (%)	54	54	51	53		
	FAT (g)	106	108	107	107		
	FAT (%)	30	33	32	32		
	PROTEIN (g)	127	97	131	118		
	PROTEIN (%)	16	13	17	15		
	Ca (mg)	1140	1166	1195	1167		
005	KCAL	3059	3053	3056	3056		
	CHO (g)	461	447	424	444	55.1	8.05
	CHO (%)	57	55	52	55		
	FAT (g)	97	102	104	101		
	FAT (%)	29	30	31	30		
	PROTEIN (g)	114	116	133	121		
	PROTEIN (%)	15	15	17	16		
	Ca (mg)	899	864	937	900		
006	KCAL	2802	2759	2810	2790		
	CHO (g)	397	399	425	407	69.9	5.82
	CHO (%)	53	54	57	55		
	FAT (g)	98	90	100	96		
	FAT (%)	31	29	32	31		
	PROTEIN (g)	111	116	81	102		
	PROTEIN (%)	16	17	11	15		
	Ca (mg)	1162	1176	1085	1141		

ID	MEASURE	MENU A	MENU B	MENU C	MEAN	FFM (kg)	g CHO/kg FFM
007	KCAL	3095	3143	3131	3123		
	CHO (g)	490	500	474	488	67.2	7.26
	CHO (%)	59	60	57	59		
	FAT (g)	93	94	107	98		
	FAT (%)	27	27	31	28		
	PROTEIN (g)	107	107	99	104		
	PROTEIN (%)	15	14	13	14		
	Ca (mg)	1213	1457	1799	1490		
008	KCAL	2533	2551	2656	2580		
	CHO (g)	391	385	389	389	61.3	6.34
	CHO (%)	57	57	57	57		
	FAT (g)	82	81	75	79		
	FAT (%)	29	28	26	28		
	PROTEIN (g)	87	96	109	97		
	PROTEIN (%)	14	15	17	15		
	Ca (mg)	803	1271	794	956		
009	KCAL	2089	2194	2183	2155		
	CHO (g)	320	315	278	304	62.6	4.86
	CHO (%)	57	54	48	53		
	FAT (g)	67	77	77	74		
	FAT (%)	29	32	32	31		
	PROTEIN (g)	71	79	113	88		
	PROTEIN (%)	14	14	21	16		
	Ca (mg)	1178	1187	1113	1159		
010	KCAL	2289	2237	2160	2229		
	CHO (g)	287	322	286	299	56.7	5.27
	CHO (%)	47	54	50	50		
	FAT (g)	87	80	80	82		
	FAT (%)	34	32	33	33		
	PROTEIN (g)	105	81	90	92		
	PROTEIN (%)	18	14	17	16		
	Ca (mg)	1038	1219	1219	1159		

Study IV

ID	MEASURE	MENU A	MENU B	MENU C	MEAN	FFM (kg)	g CHO/kg FFM
001	KCAL	2461	2345	2349	2385		
	CHO (g)	353.9	327.8	337.3	339.7	70.3	4.83
	CHO (%)	54	52	54	53.3		
	FAT (g)	79.1	94.3	79.2	84.2		
	FAT (%)	29	32	30	30.3		
	PROTEIN (g)	17	16	16	16.3		
	PROTEIN (%)	106.4	82.3	92.3	93.7		
	Ca (mg)	1269	1255	1240	1255		
002	KCAL	2013	1968	1944	1975.0		
	CHO (g)	290	284	275	283.0	72.2	3.92
	CHO (%)	54	54	53	53.7		
	FAT (g)	62.4	69.4	64.6	65.5		
	FAT (%)	28	32	30	30.0		
	PROTEIN (g)	91.4	69.7	83.1	81.4		
	PROTEIN (%)	18	14	17	16.3		
	Ca (mg)	1089	1080	1083	1084		
003	KCAL	3512.9	3525.6	3580	3539.5		
	CHO (g)	484.6	485.3	549.5	506.5	65.7	7.71
	CHO (%)	52	52	57	53.7		
	FAT (g)	124.5	130.1	109.6	121.4		
	FAT (%)	32	33	28	31.0		
	PROTEIN (g)	145.7	135.2	134.2	138.4		
	PROTEIN (%)	17	15	15	15.7		
	Ca (mg)	1249	1462	1208.1	1306.4		
004	KCAL	2053.5	2112	1925	2030.2		
	CHO (g)	317.2	317	293	309.1	78.8	3.92
	CHO (%)	58	56	57	57.0		
	FAT (g)	62.4	69.5	58.7	63.5		
	FAT (%)	27	30	27	28.0		
	PROTEIN (g)	77.4	75.3	74.3	75.7		
	PROTEIN (%)	15	14	15	14.7		
	Ca (mg)	871	777	615.6	754.5		
005	KCAL	2492	2506.9	2473.9	2490.9		
	CHO (g)	362.4	368.4	371.7	367.5	66.6	5.52
	CHO (%)	54	55	56	55.0		
	FAT (g)	79.7	84.3	88.9	84.3		
	FAT (%)	29	30	32	30.3		
	PROTEIN (g)	105.5	92.6	71.6	89.9		
	PROTEIN (%)	17	15	12	14.7		
	Ca (mg)	728.7	893	1126	916		
006	KCAL	1672	1667	1730	1689.7		
	CHO (g)	237	237	238.9	237.6	60.3	3.94
	CHO (%)	53	53	52	52.7		
	FAT (g)	48.5	50.9	55.2	51.5		
	FAT (%)	26	27	29	27.3		
	PROTEIN (g)	86.4	79.9	84.1	83.5		
	PROTEIN (%)	21	19	19	19.7		
	Ca (mg)	831	801	802.3	811.4		

ID	MEASURE	MENU A	MENU B	MENU C	MEAN	FFM (kg)	g CHO/kg FFM
007	KCAL	3059	3053	3056	3055.9		
	CHO (g)	460.8	446.5	423.9	443.7	56.4	7.87
	CHO (%)	57	55	52	54.7		
	FAT (g)	97	102.4	103.8	101.1		
	FAT (%)	29	30	31	30.0		
	PROTEIN (g)	114.1	115.8	133.1	121.0		
	PROTEIN (%)	15	15	17	15.7		
	Ca (mg)	899	864	937	900		
008	KCAL	2050	2073	2122.9	2082.0		
	CHO (g)	297.7	318.8	312.2	309.6	64.3	4.81
	CHO (%)	54	58	55	55.7		
	FAT (g)	76.5	62.9	66.6	68.7		
	FAT (%)	30	27	28	28.3		
	PROTEIN (g)	82.6	78.3	88	83.0		
	PROTEIN (%)	16	12	17	15.0		
	Ca (mg)	793	774	743	770.0		
009	KCAL	3113	3080	2995	3062.7		
	CHO (g)	451.9	438.2	427.8	439.3	69.7	6.30
	CHO (%)	55	53	54	54.0		
	FAT (g)	97.6	110.5	99.1	102.4		
	FAT (%)	29	32	30	30.3		
	PROTEIN (g)	125.7	111.6	124.4	120.6		
	PROTEIN (%)	16	14	17	15.7		
	Ca (mg)	717	894	746	786		
010	KCAL	2533	2551	2656	2580		
	CHO (g)	391.1	385.4	389.3	389	64.4	6.03
	CHO (%)	57	57	57	57		
	FAT (g)	82.1	80.6	74.9	79		
	FAT (%)	29	28	26	28		
	PROTEIN (g)	86.6	95.5	108.6	97		
	PROTEIN (%)	14	15	17	15		
	Ca (mg)	803	1271	794	956		

Appendix B

Relationships between changes in phosphate (PO_4) and parathyroid hormone (PTH) during exercise in Studies I-IV

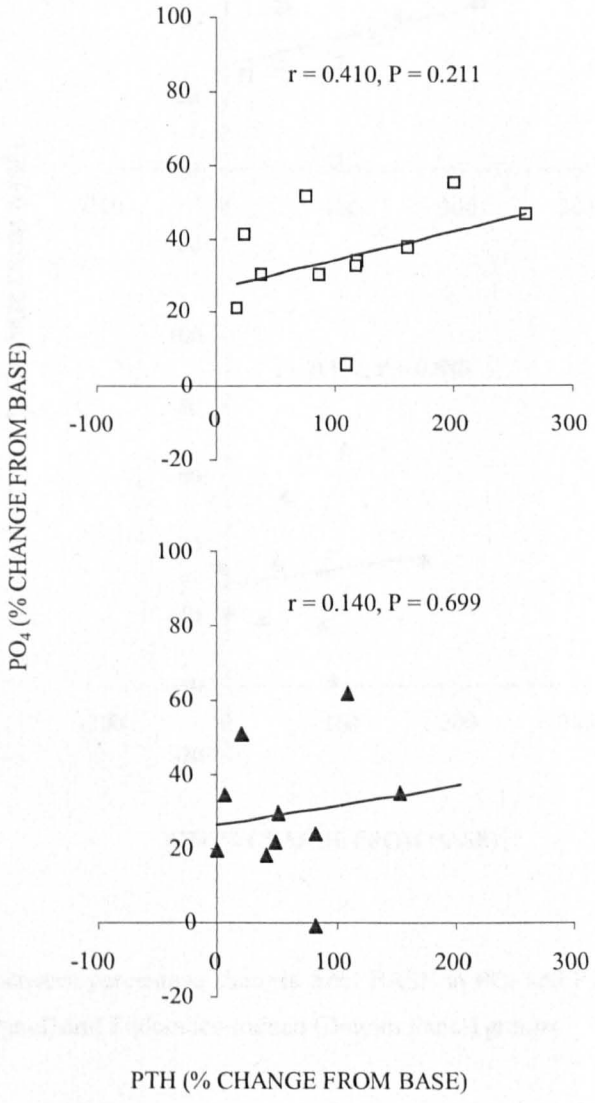

Figure shows relationships between percentage changes from BASE in PO_4 and PTH at EX40 in Study I in the Recreationally-active (Top Panel) and Endurance-trained (Bottom Panel) groups.

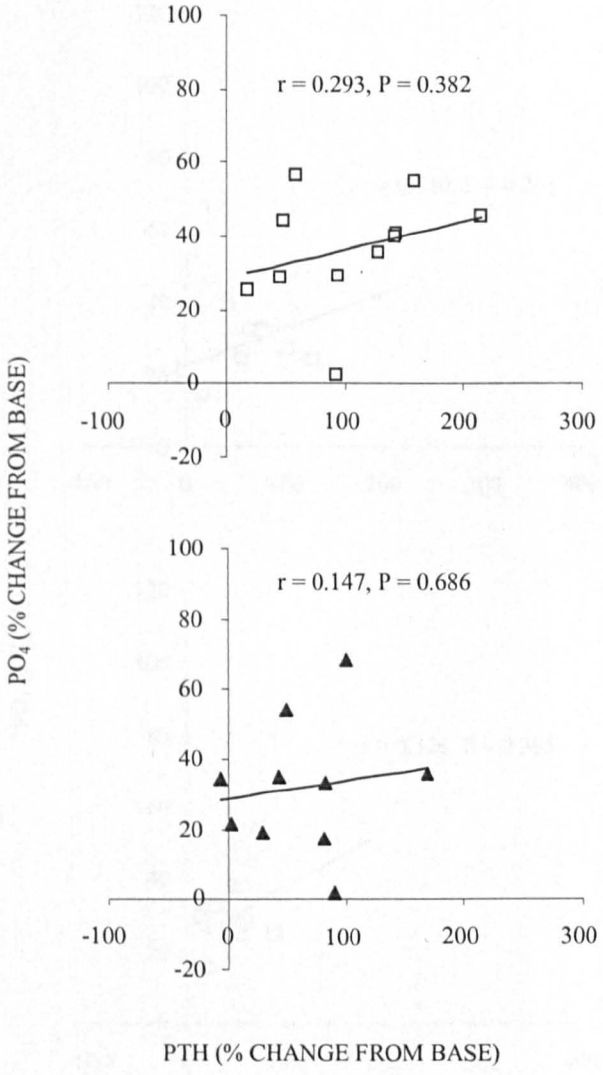

Figure shows relationships between percentage changes from BASE in PO_4 and PTH at EX60 in Study I in the Recreationally-active (Top Panel) and Endurance-trained (Bottom Panel) groups.

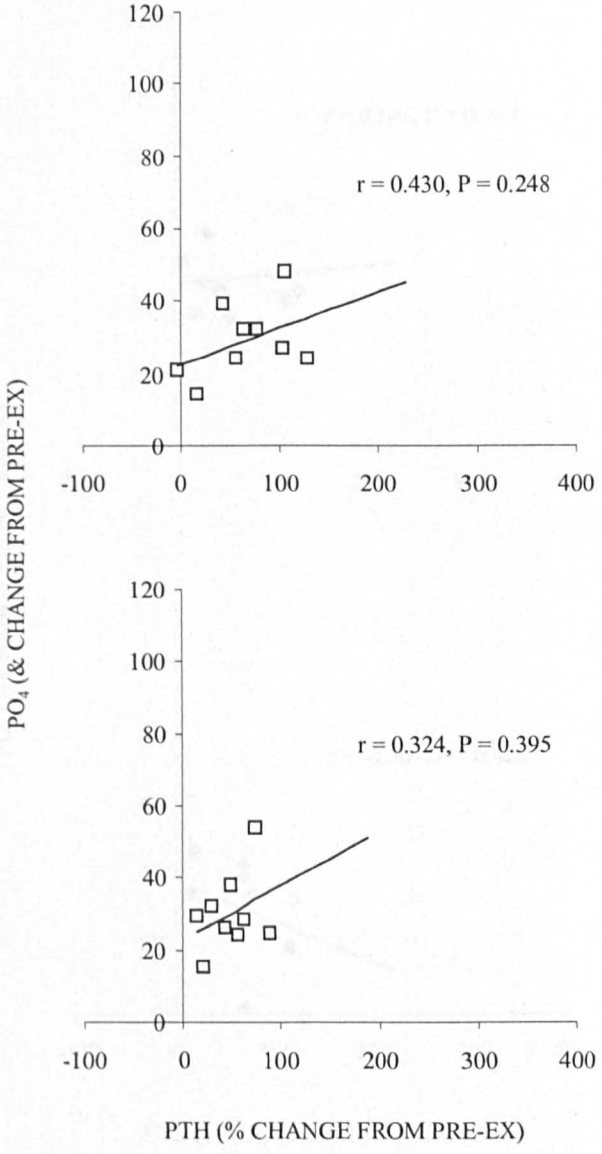

Figure shows relationships between percentage changes in PO_4 and PTH measured from pre-exercise (PRE-EX) to immediately post-exercise during L-ExA (Top Panel) and L-ExB (Bottom Panel) when the data from Subject 7 is excluded. Note that the significant correlation for L-ExA, evident when data from Subject 7 was included, is no longer present.

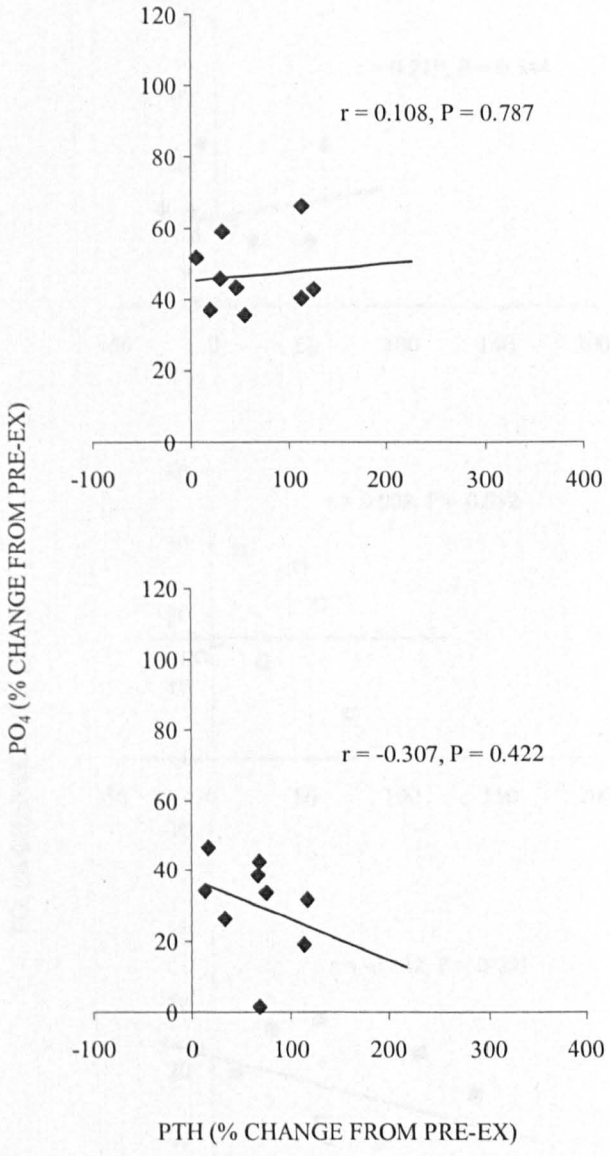

Figure shows relationships between percentage changes in PO_4 and PTH measured from pre-exercise (PRE-EX) to immediately post-exercise during S-ExA (Top Panel) and S-ExB (Bottom Panel) when the data from Subject 10 is excluded. Note that the significant correlation for S-ExA and the borderline correlation for L-ExB, evident when data from Subject 10 was included, are no longer present.

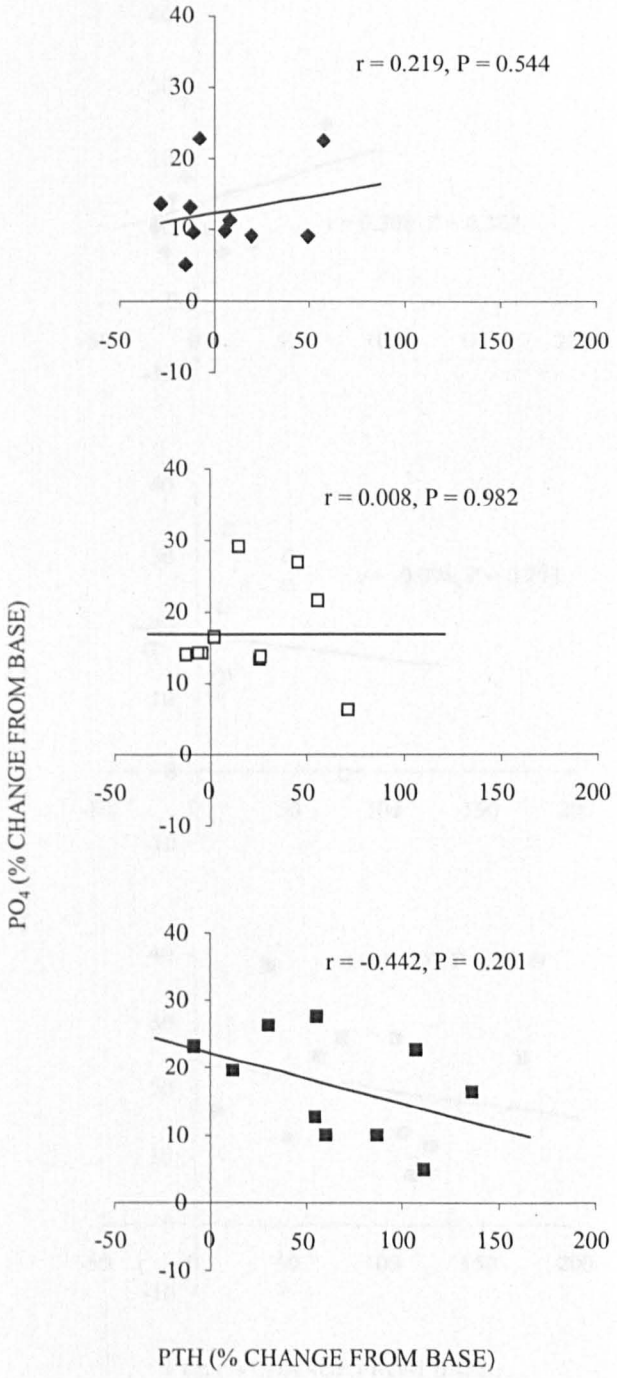

Figure shows relationships between percentage changes from BASE in PO_4 and PTH at EX40 in Study III in the LOW (Top Panel), MOD (Middle Panel) and HIGH (Bottom Panel) conditions.

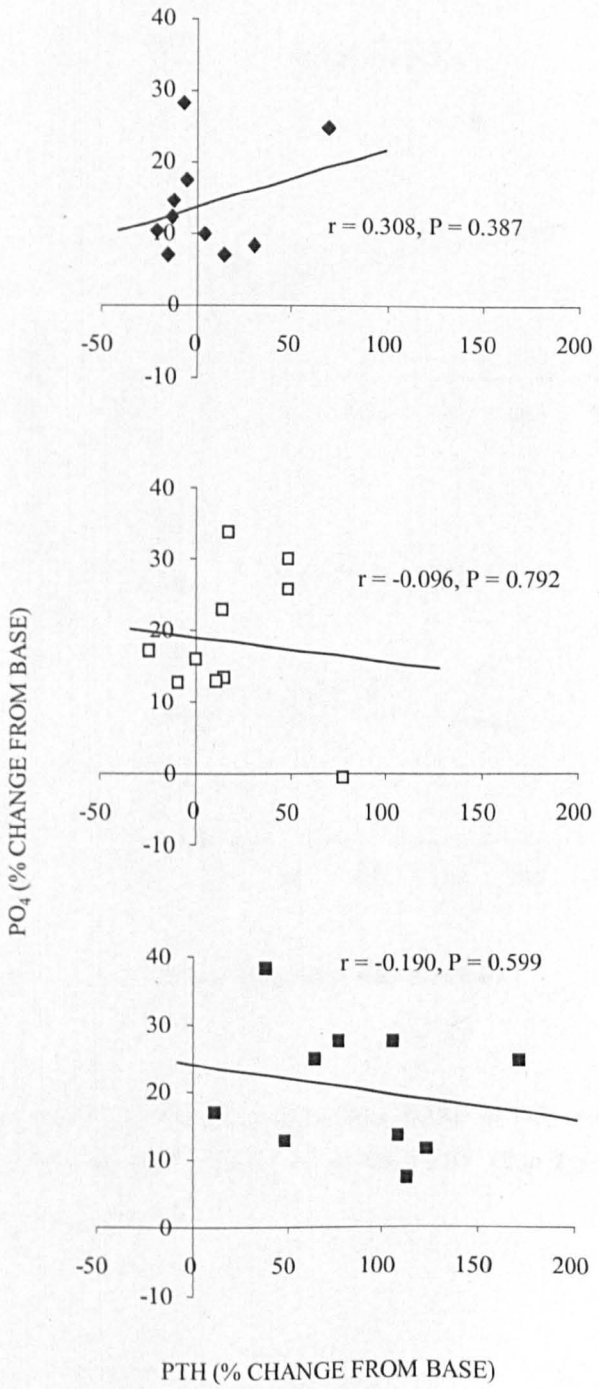

Figure shows relationships between percentage changes from BASE in PO_4 and PTH at EX60 in Study III in the LOW (Top Panel), MOD (Middle Panel) and HIGH (Bottom Panel) conditions.

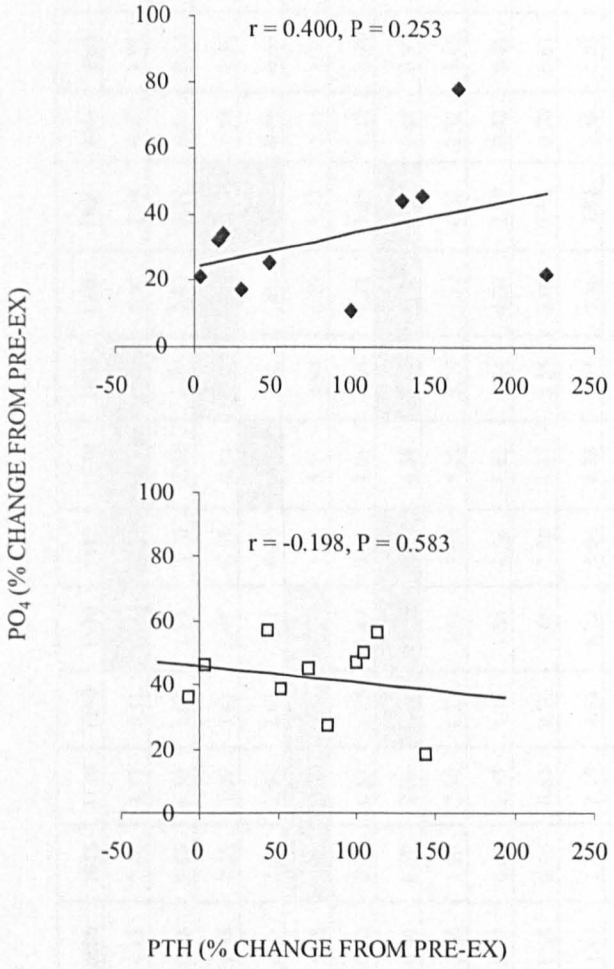

Figure shows relationships between percentage changes from BASE in PO_4 and PTH measured from 1015 h (PRE-EX) to immediately post-exercise in Study IV in the FAST (Top Panel) and FED (Bottom Panel) conditions.

Appendix C

Raw IL-6 and IL-1ra data from Study II

IL-6 data in the LONG condition

Condition	ID	0800	1415	1530	1630	1730	1830	0800	1015	1130	1230	1330	1415	1530	1630	1730	1830	FU1	FU2	FU3	FU4
LONG	005	0.89	26.60	12.90	20.85	23.82	17.91	4.13	2.80	4.83	5.51	20.85	13.59	11.28	9.80	5.67	2.08	4.47	1.91	0.43	0.64
	007	0.72	2.04	6.04	12.73	13.49	10.74	0.65	0.52	0.59	1.04	1.88	2.70	2.91	3.09	2.97	2.41	0.60	0.64	0.62	0.60
	008	1.26	1.18	5.85	16.26	9.91	10.10	1.04	1.07	1.89	3.41	3.47	6.13	7.11	13.20	21.63	22.10	1.58	1.19	0.89	0.98
	010	0.78	0.49	4.01	2.77	3.14	3.02	ns	3.07	2.94	2.63	7.21	8.05	12.72	ns	ns	10.80	0.80	0.61	0.66	0.97
	011	3.09	1.72	9.10	6.44	6.61	5.96	5.88	11.43	15.17	16.80	33.78	1.45	5.77	4.94	5.19	6.12	1.11	1.75	0.89	0.91
	012	0.93	0.46	3.44	2.22	5.48	1.44	0.90	0.96	0.57	1.35	1.47	2.51	4.01	4.04	4.22	3.20	1.11	0.78	4.85	0.80
	013	0.30	0.36	4.06	2.27	3.36	3.38	0.60	0.59	2.04	10.19	11.02	14.84	4.38	20.34	16.35	19.50	0.68	0.97	0.74	0.58
	014	1.84	1.91	6.20	10.18	8.68	9.32	2.96	1.90	2.30	2.22	3.21	3.08	5.27	5.29	3.77	6.02	2.32	1.42	1.39	1.63
	015	0.29	0.29	6.61	4.20	4.55	4.35	0.77	0.66	0.93	1.26	1.84	2.53	4.82	3.24	4.37	2.19	0.42	0.41	0.39	0.51
	017	0.47	0.27	7.05	3.43	3.36	3.18	0.56	0.50	0.63	0.76	2.46	1.56	5.27	4.55	3.00	3.80	0.70	0.61	0.39	0.38
	Mean	**1.06**	**3.53**	**6.53**	**8.14**	**8.24**	**6.94**	**1.94**	**2.35**	**3.19**	**4.52**	**8.72**	**5.64**	**6.35**	**7.61**	**7.46**	**7.82**	**1.38**	**1.03**	**1.13**	**0.80**
	SD	**0.85**	**8.14**	**2.79**	**6.60**	**6.42**	**5.06**	**1.94**	**3.33**	**4.41**	**5.17**	**10.68**	**4.98**	**3.19**	**5.83**	**6.73**	**7.35**	**1.22**	**0.52**	**1.34**	**0.36**

Data points considered to be outliers are highlighted in grey

IL-6 data in the SHORT condition

Condition	ID	0800	1415	1530	1630	1730	1830	0800	1015	1130	1230	1330	1415	1530	1630	1730	1830	FU1	FU2	FU3	FU4
SHORT	005	0.51	0.35	0.38	0.44	0.60	1.08	0.44	1.04	5.71	4.96	4.81	5.30	11.51	9.10	3.05	2.68	2.56	0.47	0.68	1.28
	007	0.61	0.56	0.63	1.27	1.78	3.93	0.44	1.28	3.41	4.13	2.38	2.43	6.77	6.37	8.27	14.10	1.09	0.87	0.91	1.89
	008	0.99	0.71	1.24	3.85	4.13	7.30	1.17	2.13	9.55	20.88	9.65	16.80	21.12	28.38	24.18	37.20	8.73	0.95	0.77	0.87
	010	0.86	0.53	0.85	1.09	1.43	1.63	1.25	1.57	8.71	11.92	13.84	1.54	6.90	3.16	2.86	3.54	1.20	0.61	0.70	0.65
	011	11.48	12.86	12.26	11.87	10.79	11.00	3.30	2.66	15.56	14.59	14.22	3.67	33.24	25.02	22.14	18.96	14.94	8.59	10.00	6.01
	012	0.65	0.29	0.38	0.57	0.51	0.65	0.41	3.09	5.00	7.02	15.57	1.98	7.15	6.81	9.62	6.73	0.90	1.48	0.53	0.44
	013	0.34	0.34	1.10	3.45	15.39	11.39	0.63	1.01	4.47	6.06	7.21	5.12	5.26	8.63	11.15	13.77	1.13	0.54	0.40	0.42
	014	2.98	3.21	3.77	5.50	6.23	5.65	4.23	4.08	6.73	6.76	7.15	5.52	7.77	6.05	5.25	6.72	4.52	4.18	4.35	3.58
	015	0.43	0.35	0.45	0.68	0.45	0.95	0.41	4.48	5.26	5.02	4.55	5.76	11.98	11.17	5.80	5.62	1.60	0.57	0.36	0.33
	017	0.48	0.37	0.78	1.33	1.02	1.07	0.69	0.60	6.92	4.50	4.19	5.15	10.36	9.68	10.12	9.16	2.70	1.63	0.66	0.58
	Mean	**1.93**	**1.96**	**2.18**	**3.01**	**4.23**	**4.47**	**1.30**	**2.19**	**7.13**	**8.58**	**8.36**	**5.33**	**12.21**	**11.44**	**10.24**	**11.85**	**3.94**	**1.99**	**1.94**	**1.61**
	SD	**3.44**	**3.93**	**3.68**	**3.55**	**5.13**	**4.19**	**1.35**	**1.35**	**3.51**	**5.51**	**4.73**	**4.33**	**8.67**	**8.38**	**7.39**	**10.29**	**4.55**	**2.57**	**3.07**	**1.84**

Data points considered to be outliers are highlighted in grey

IL-1ra data in the LONG condition

Condition	ID	0800	1415	1530	1630	1730	1830	0800	1015	1130	1230	1330	1415	1530	1630	1730	1830	FU1	FU2	FU3	FU4
LONG	005	133	164	286	805	827	699	202	181	176	162	209	192	ns	823	628	434	184	143	149	136
	007	280	188	299	560	568	442	242	193	197	237	195	231	221	361	392	292	176	176	137	202
	008	292	196	329	489	443	428	229	200	209	219	203	202	284	512	777	921	243	177	171	211
	010	179	196	272	542	465	359	ns	278	275	269	281	273	364	ns	ns	370	238	196	191	182
	011	496	495	593	599	613	587	412	430	430	423	680	402	483	516	514	476	395	428	387	353
	012	182	168	267	265	362	238	209	168	139	168	158	201	209	389	382	284	176	193	179	122
	013	271	146	160	191	200	202	242	169	150	ns	165	157	207	205	207	182	165	160	157	157
	014	218	178	361	1187	850	525	339	249	212	193	237	223	391	413	401	394	301	271	280	225
	015	161	86	261	2145	1806	910	193	153	153	158	145	168	235	1334	1007	605	138	116	175	108
	017	188	128	288	831	689	456	412	205	214	268	269	205	335	653	558	435	160	166	245	225
	Mean	**240**	**194**	**312**	**761**	**682**	**485**	**276**	**223**	**216**	**233**	**254**	**226**	**303**	**579**	**540**	**439**	**218**	**203**	**207**	**192**
	SD	**105**	**111**	**112**	**564**	**443**	**211**	**88**	**82**	**85**	**83**	**156**	**70**	**97**	**334**	**240**	**206**	**79**	**89**	**77**	**71**

Data points considered to be outliers are highlighted in grey

IL-1ra data in the SHORT condition

Condition	ID	0800	1415	1530	1630	1730	1830	0800	1015	1130	1230	1330	1415	1530	1630	1730	1830	FU1	FU2	FU3	FU4
SHORT	005	161	209	193	189	172	140	151	135	153	301	326	299	335	365	337	306	179	144	122	137
	007	160	154	200	158	181	169	179	173	252	246	353	329	356	343	333	370	182	165	171	177
	008	231	208	196	226	245	226	232	185	270	337	367	334	397	812	1260	1044	279	180	177	193
	010	151	150	171	177	179	179	213	193	295	673	702	286	383	329	314	316	237	217	187	172
	011	1387	2781	2819	2543	1953	1868	631	641	836	1141	1043	829	948	1116	1300	1097	686	685	611	571
	012	142	120	145	153	131	131	192	156	171	198	318	144	208	240	223	226	134	138	116	79
	013	152	128	145	141	146	126	260	128	172	140	149	171	208	187	190	205	167	96	107	ns
	014	233	179	268	225	191	215	336	172	278	305	356	358	458	499	504	479	331	379	280	374
	015	87	121	110	138	123	95	111	92	163	348	333	256	307	675	622	464	219	151	118	78
	017	175	145	148	336	165	187	278	159	307	576	668	522	634	763	696	580	425	147	169	218
	Mean	**288**	**419**	**439**	**429**	**349**	**334**	**258**	**203**	**290**	**426**	**462**	**353**	**423**	**533**	**578**	**509**	**284**	**230**	**206**	**222**
	SD	**389**	**830**	**837**	**745**	**565**	**541**	**146**	**157**	**201**	**299**	**263**	**197**	**221**	**299**	**404**	**318**	**166**	**177**	**151**	**157**

Data points considered to be outliers are highlighted in grey

The Effect of Training Status on the Metabolic Response of Bone to an Acute Bout of Exhaustive Treadmill Running

Jonathan P. R. Scott, Craig Sale, Julie P. Greeves, Anna Casey, John Dutton, and William D. Fraser

Human Protection and Performance Enhancement (J.P.R.S., A.C.), QinetiQ, Farnborough GU14 0LX, United Kingdom; School of Science and Technology (C.S.), Nottingham Trent University, Nottingham NG1, United Kingdom; Plans Branch (J.P.G.), Headquarters Army Recruiting and Training Division, Upavon SN9 6BE, United Kingdom; and Unit of Clinical Chemistry (J.D., W.D.F.), School of Clinical Sciences, University of Liverpool, Liverpool L69, United Kingdom

Context: Strenuous exercise increases bone resorption but not formation. The effect of improved training status is unknown.

Objective: Our objective was to examine the metabolic response of bone to strenuous running in recreationally active (RA) and endurance-trained (ET) men.

Design: Eleven RA, 10 ET, and 10 control (CON) subjects completed one 8-d trial. On d 4, RA and ET completed an exhaustive treadmill run. Blood was obtained at baseline (BASE), during exercise, during 2 h of recovery, and on four follow-up (FU) days (FU1–FU4). CON rested throughout, providing blood samples at BASE and on FU1–FU4. Markers of bone resorption [C-terminal telopeptide region of collagen type 1 (β-CTX)] and bone formation [N-terminal propeptides of procollagen type 1 (P1NP) and bone alkaline phosphatase (ALP)], osteoprotegerin (OPG), PTH, albumin-adjusted calcium (ACa), and phosphate (PO_4) were measured.

Results: There were no significant differences between ET and RA and no changes in CON for any variable. Exercise increased β-CTX at FU1–FU4 ($P < 0.001$) but had no effect on P1NP or bone ALP. OPG was increased after 20 min of exercise ($P < 0.001$) and remained elevated at FU1 ($P < 0.001$).

PTH, ACa, and PO_4 were increased throughout exercise ($P < 0.01$). ACa and PO_4 remained elevated in the 2 h after exercise ($P < 0.001$), whereas PTH was lower than BASE from 1–2 h after exercise ($P < 0.001$).

Conclusion: After acute, exhaustive running, bone resorption but not formation was increased for 4 d in RA and ET men. The increased bone resorption might be related to the increase in PTH, whereas increased OPG might be a compensatory response to increased bone resorption. Training status did not significantly affect the metabolic response of bone to exhaustive running. **(*J Clin Endocrinol Metab* 95: 3918–3925, 2010)**

Exercise has been associated with stress fracture injury in military recruits (1) and both stress fractures (2) and reduced spinal bone mineral density (BMD) in endurance-trained (ET) athletes (3). The net effects of changes in bone resorption and formation, which are normally coupled in a basic multicellular unit, have been implicated in both stress fractures (4) and changes in BMD (5). Short-term changes in bone turnover markers have been re-

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Abbreviations: ACa, Albumin-adjusted calcium; ALP, alkaline phosphatase; AUC_I, area under the curve with respect to increase; BASE, baseline; BMD, bone mineral density; β-CTX, C-terminal telopeptide region of collagen type 1; CV, coefficient of variation; EE, end of intermittent exhaustive exercise; ET, endurance-trained; FD, fixed-duration exercise; LMM, linear mixed model; OC, osteocalcin; OPG, osteoprotegerin; P1NP, N-terminal propeptides of procollagen type 1; R0.5, 0.5 h of recovery; RA, recreationally active; RANK-L, receptor activator of nuclear factor-κB ligand; VO_{2max}, maximal O_2 consumption.

TABLE 1. Subject characteristics of the RA, ET, and CON groups

	RA	ET	CON
n	11	10	10
Age (yr)	30 ± 3	31 ± 3[e]	26 ± 3[a]
Height (m)	1.84 ± 0.06	1.73 ± 0.06[c,d]	1.79 ± 0.05
Body mass (kg)	84.4 ± 8.4	69.8 ± 6.6[b,d]	77.1 ± 7.0[b]
BMI (kg/m^2)	25.0 ± 2.9	23.3 ± 1.6	24.0 ± 2.2
VO_{2max} (ml/min · kg)	55.5 ± 6.8	67.9 ± 6.1[c,f]	53.7 ± 3.0
Physical activity (h/wk)	1.4 ± 0.4	4.5 ± 1.3[c,f]	1.3 ± 0.5

Values are mean ± 1 SD. BMI, Body mass index.

[a] Different ($P < 0.05$) from RA.

[b] Different ($P < 0.01$) from RA.

[c] Different ($P < 0.001$) from RA.

[d] Different ($P < 0.05$) from CON.

[e] Different ($P < 0.01$) from CON.

[f] Different ($P < 0.001$) from CON.

ported in response to acute weight-bearing (6–8), non-weight-bearing (9–11), and resistance (12) exercise.

Guillemant *et al.* (10) reported a 45–50% increase in C-terminal telopeptide region of collagen type 1 (β-CTX) with no change in bone alkaline phosphatase (ALP) for up to 2 h after 60 min of cycling. Others have shown increased β-CTX and decreased osteocalcin (OC) immediately after 246 km of running (6). These data suggest that strenuous exercise may induce osteoclastic activity that is not accompanied by an associated increase in osteoblastic activity, at least in the short term.

Two studies exist where volunteers of different training status have undergone the same acute exercise protocol (9, 13). Nishiyama *et al.* (13) reported higher basal OC in volleyball players and a more rapid increase in OC after 30 min of running but did not measure any markers of bone resorption.

Herrmann *et al.* (9) studied bone markers in male athletes and sedentary controls performing 60 min of cycling using standardized exercise intensities. However, this study made no statistical comparison between the groups and did not standardize the time of day or the nutritional status of subjects when collecting blood, both of which are known to affect bone marker concentrations, particularly β-CTX (14, 15).

To date, no controlled laboratory studies using more specific bone turnover markers have investigated whether the increase in bone resorption persists on the days that follow weight-bearing exercise. Additionally, no controlled studies have systematically examined the effect of training status on the bone turnover marker response to acute exercise.

The aim of the present study was to examine the effect of training status on specific bone turnover markers for 4 d after an acute bout of strenuous running. It was hypothesized that a single bout of exhaustive running would increase markers of bone resorption but not bone formation. It was further hypothesized that endurance training would modify the bone turnover marker response to exercise.

Subjects and Methods

Subjects

Twenty-one recreationally active (RA) [11 in the exercise group and 10 in the control (CON) group] and 10 ET males were recruited to the study, which was approved by the QinetiQ Research Ethics Committee (Table 1). Subjects provided written informed consent.

Subjects were included if they were nonsmokers, had not suffered a bone fracture in the previous 12 months, were free from musculoskeletal injury, and were not taking any medication or suffering from any condition known to affect bone metabolism. Compliance with these inclusion criteria was confirmed from a medical screening questionnaire and a medical examination. Subjects were considered RA if they completed two to three exercise sessions per week including at least one run, but performed no more than 2 h/wk of exercise and were not involved in an organized training program (Table 1). Subjects in ET were required to have been running for a minimum of 2 yr without a significant break and to have completed a measured 10-km distance in less than 40 min within the previous 4 wk.

Design

Subjects completed two preliminary visits for medical screening, habituation, and measurement of maximal O_2 consumption (VO_{2max}). Subjects then completed one 8-d experimental trial. On d 1–3, subjects refrained from physical activity and followed a prescribed diet. On d 4, ET and RA performed an acute, exhaustive bout of treadmill running while CON rested. On d 5–8, all subjects refrained from physical activity, followed a prescribed diet, and attended the laboratory for follow-up analyses.

Pretrial measurements

Dietary analysis

Subjects completed a 3-d, weighed-food diary consisting of two weekdays and one weekend day to calculate habitual daily

energy intake and dietary macronutrient composition (Microdiet V2; Downlee Systems Ltd., Chapel-en-le-Frith, UK).

Assessment of cardiorespiratory responses to running and aerobic power

Subjects completed an incremental, submaximal treadmill (XELG 70 ERGO; Woodway, Waukesha, WI) run consisting of four 5-min stages, with 60-sec expired air samples collected in the final minute of each stage. After a 30-min rest, subjects completed a discontinuous, incremental exercise test to exhaustion to establish VO_{2max} (16). The results of the two tests were used to estimate the treadmill velocity corresponding to 65 and 70% VO_{2max}.

Experimental dietary provision

A diet containing 8 g carbohydrate/kg fat-free mass · d and isocaloric with their habitual diet was designed for each subject based on individual dietary habits. Subjects provided their own food and were given menus, calibrated weighing scales, and instructions concerning the quantity, preparation, and timing of meals. Deviations from prescribed diets were recorded in a diary and confirmed verbally on d 4–8. Compliance was good in all three groups.

Trial procedures

Days 1–3

All subjects refrained from physical activity and consumed their experimental diets.

Day 4 (RA and ET)

RA and ET subjects arrived overnight fasted at 0730 h, voided, and had their nude body mass measured. Subjects adopted a semirecumbent position and placed their left hand into a hand warming unit (Medical Physics, Nottingham, UK) for 20 min. A cannula was inserted into a vein in the back of the hand and was kept patent with an isotonic saline solution (0.9% NaCl) before a baseline (BASE) blood sample was collected for the measurement of all biochemical markers. Subjects then completed a 60-min run at 65% of VO_{2max} [fixed-duration exercise (FD)]. After a 15-min seated rest, subjects ran to exhaustion at 70% VO_{2max}, rested for 5 min, and then resumed exercise. This work-rest pattern was repeated until subjects were not able to complete 5 min of continuous running. Rest breaks were subsequently reduced to 1 min, and the work-rest pattern was repeated until subjects were not able to complete 2 min of continuous running, at which point exercise was terminated. This work-rest protocol will be referred to as intermittent exhaustive exercise. Blood samples were collected after 20, 40, and 60 min of FD (FD20, FD40, and FD60, respectively) and again at the end of intermittent exhaustive exercise (EE) for the measurement of glucose, lactate, osteoprotegerin (OPG), PTH, albumin-adjusted calcium (ACa), and phosphate (PO_4) only (Fig. 1).

At EE, subjects adopted a semirecumbent position and returned their hand to the warming unit. Further blood samples were drawn at 0.5 h (R0.5), 1 h (R1.0), 1.5 h (R1.5), and 2 h (R2.0) of recovery (Fig. 1). Subjects remained fasted until the final blood sample was drawn after R2.0.

Day 4 (CON)

Subjects arrived overnight fasted at 0730 h, voided, and had their nude body mass measured. A blood sample was drawn by venipuncture between 0800 and 0830 h for the measurement of bone turnover markers.

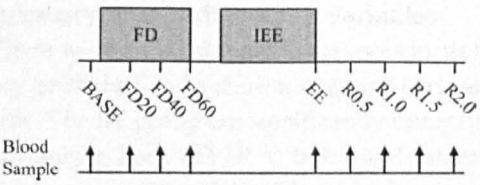

FIG. 1. Outline of exercise protocol and blood sampling schedule on d 4 of the experimental condition for the RA and ET groups. Subjects remained fasted until after the final blood sample was drawn at R2.0. *Gray boxes* denote exercise; FD was 60 min at 65% VO_{2max}. IEE, Intermittent exhaustive exercise at 70% VO_{2max}.

Days 5–8 (FU1–FU4, all subjects)

All subjects arrived overnight fasted, voided, and had nude body mass measured. Between 0800 and 0830 h, blood was drawn by venipuncture for the measurement of all biochemical markers.

Biochemical analysis

β-CTX was measured in EDTA plasma using an electrochemiluminescent immunoassay on an Elecsys 2010 immunoanalyzer (Roche, Lewes, UK). Interassay coefficient of variation (CV) was less than 8% from 0.2–1.5 μg/liter. The assay sensitivity (replicates of the zero standard) was 0.01 μg/liter. N-terminal propeptides of procollagen type 1 (P1NP) was measured in EDTA plasma by RIA supplied by Orion Diagnostica (Espoo, Finland). This assay has a sensitivity of 4 μg/liter established from precision profiles (22% CV of duplicates) and an interassay CV of 3.5–5.4% from 10–250 μg/liter. Bone ALP was measured in serum using a commercial immunometric assay (Metra Biosystems, Oxford, UK) with a sensitivity of 0.7 U/liter and a CV of less than 8% from 12–100 U/liter. OPG was measured in EDTA plasma using a commercial solid-phase ELISA that measures both free OPG and that complexed with receptor activator of nuclear factor-κB ligand (RANK-L) (IDS Boldon, Tyne and Wear, UK). The assay has a detection limit of 0.14 pmol/liter and inter-/intraassay CV of less than 10% from 1–30 pmol/liter.

PTH was measured in serum using an immunometric assay (Nichols Institute, San Juan Capistrano, CA) with a detection limit of 0.5 pmol/liter and inter- and intraassay CV of less than 5% from 1–40 pmol/liter. Calcium (range of measurement in serum of 0.05–5.00 mmol/liter), albumin (range of measurement in serum of 10–70 g/liter), and PO_4 (range of measurement in serum of 0.10–6.46 mmol/liter) were measured in serum using standard commercial assays supplied by Roche and performed on a Roche modular analytical system. Glucose and lactate were measured immediately in whole blood in duplicate (Yellow Springs Instruments 2300 Stat Plus; YSI Inc., Yellow Springs, OH).

Statistical analysis

All data are presented as mean ± 1 SD. Statistical significance was accepted at $P < 0.05$. One-way ANOVA was used to compare VO_{2max}, habitual and experimental dietary variables, and BASE biochemistry between groups, with Newman Keuls *post hoc* tests performed where appropriate. Student's *t* tests for unpaired data and paired data were used to compare exercise variables between the RA and ET groups and habitual with experimental dietary data within all three groups, respectively.

Biochemical data were analyzed using a linear mixed model (LMM) ANOVA.

Where datasets did not satisfy assumptions of the LMM (P1NP, OPG, PTH, and ACa), normality and homogeneity were achieved after log transformations. Where there was a significant main effect of time but no significant group × time interaction, pooled mean data at each subsequent time point was compared against BASE using a Dunnett's test. When the group × time interaction was significant, each subsequent time point within each group was compared against BASE using a Dunnett's test, and groups were compared with each other at all time points using a Newman Keuls test. The area under the curve with respect to increase (AUC_I) was calculated (17) for bone turnover markers derived from the trapezoid formula (18) and the three groups compared with a one-way ANOVA and a Newman Keuls test.

Results

Subject characteristics

Subject characteristics in the RA, ET, and CON groups are shown in Table 1.

Dietary analysis

Subjects in the ET group reported consuming a greater quantity of carbohydrate relative to fat-free mass than both RA and CON ($P < 0.05$), but there were no other differences in habitual diets between the three groups (Table 2). Daily calcium intake reported by all subjects exceeded 700 mg/d.

The energy content of the experimental diet was not significantly different from habitual energy intake in any group (RA, $P = 0.107$; ET, $P = 0.368$; CON, $P = 0.401$), and there were no significant differences between the three groups for any experimental diet variable (P values from 0.095–0.935, data not shown).

BASE biochemistry

There were no significant differences between the three groups for any variable (P values from 0.071–0.892, data not shown).

Respiratory and performance variables

There were no significant differences in VO_2 or respiratory exchange ratio during exercise between the two groups. The ET group ran significantly faster than the RA group during both FD ($P < 0.05$) and intermittent exhaustive exercise ($P < 0.05$). The total distance covered by ET was about 50% greater ($P < 0.05$) than RA (29.1 ± 3.8 *vs.* 19.8 ± 3.5 km). Body mass did not change significantly in any of the groups.

Blood glucose and lactate

Blood glucose was increased ($P < 0.001$) from BASE in both RA and ET throughout FD (Fig. 2A). At EE, blood glucose was lower compared with BASE in RA and ET and remained so until 2 h after exercise ($P < 0.001$). The blood glucose response to exercise was not significantly different between RA and ET.

Blood lactate concentrations were increased from BASE in both groups during FD, at EE, and up to 30 min after exercise ($P < 0.05$) (Fig. 2B). Lactate concentrations were higher in RA compared with ET at FD20 ($P < 0.05$) and FD40 ($P < 0.05$). There were no other significant differences.

Bone resorption markers

Both a significant effect of time ($P < 0.001$) and a significant group × time interaction ($P < 0.05$) were observed for β-CTX. There was no significant change in β-CTX concentrations in CON from BASE to FU4 (Fig. 3A). In RA and ET, β-CTX concentrations were significantly increased from BASE at FU1 (RA, $P < 0.001$; ET, $P < 0.01$), FU2 (RA and ET, $P < 0.001$), FU3 (RA and ET, $P < 0.01$), and FU4 (RA, $P < 0.001$; ET, $P < 0.05$).

$AUC_{I(BASE-FU4)}$ for β-CTX was significantly greater ($P < 0.01$) in RA and ET compared with CON (Fig. 3D), but there was no significant difference between RA and ET.

TABLE 2. Habitual energy intake, macronutrient composition, and dietary calcium content of the RA, ET, and CON groups

	RA	ET	CON
Energy (MJ)	11.0 ± 1.5	11.6 ± 2.6	10.5 ± 1.9
CHO (g)	324 ± 59	383 ± 65	328 ± 70
CHO (g/kg FFM)	5.4 ± 0.9	6.7 ± 1.3[a,b]	5.4 ± 0.8
CHO (% of total energy)	47.3 ± 6.1	53.2 ± 6.5	49.4 ± 5.7
Fat (% of total energy)	30.9 ± 4.5	27.2 ± 7.0	30.2 ± 6.5
Protein (% of total energy)	18.1 ± 2.3	17.8 ± 3.5	17.4 ± 2.5
Calcium (mg/d)	1210 ± 280 (852–1823)	1184 ± 379 (708–1963)	1044 ± 264 (719–1556)

Values are mean ± 1 SD unless otherwise stated (ranges in *parentheses*). CHO, Carbohydrate; FFM, fat-free mass.

[a] Different ($P < 0.05$) from RA.

[b] Different ($P < 0.05$) from CON.

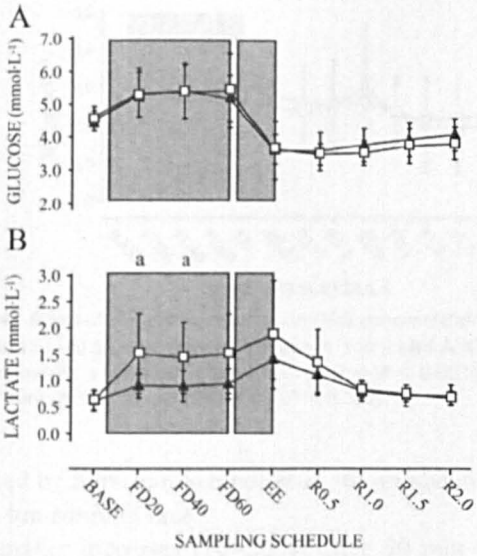

FIG. 2. Effect of exhaustive exercise on blood glucose (A) and blood lactate (B) concentrations in the RA (□) and ET (▲) groups. Values are mean ± 1 SD. LMM ANOVA revealed the following: a significant main effect of time ($P < 0.001$) but no significant group × time interaction ($P = 0.754$) for glucose and a significant main effect of time ($P < 0.01$) and a significant group × time interaction ($P < 0.01$) for lactate. a, Different ($P < 0.05$) from RA.

Bone formation markers

Both ANOVA and $AUC_{I(BASE-FU4)}$ analyses indicated that there was no significant effect of exercise on P1NP (Fig. 3, B and D) or bone ALP (Fig. 3, C and D).

Osteoprotegerin

OPG was significantly elevated from BASE in both groups during FD ($P < 0.001$), at EE ($P < 0.001$), and for 2 h after exercise ($P < 0.001$) (Fig. 4). OPG remained significantly elevated from BASE at FU1 ($P < 0.001$) in both groups, but there were no differences from BASE thereafter.

Calcium metabolism

PTH was significantly increased from BASE during FD ($P < 0.001$) and at EE in both groups ($P < 0.001$) (Fig. 5A). After 30 min of recovery, PTH concentrations had returned to BASE and were significantly lower than BASE at R1.0, R1.5, and R2.0 ($P < 0.001$). PTH was similar to BASE on the four follow-up days, and there were no significant differences between groups.

Both a significant effect of time ($P < 0.001$) and a significant group × time interaction ($P < 0.001$) were observed for ACa. In RA, the ACa concentration was increased during FD ($P < 0.01$), at EE ($P < 0.001$), and for 2 h after exercise ($P < 0.001$) (Fig. 5B). In ET, ACa was significantly increased from BASE at FD40 ($P < 0.01$), FD60 ($P < 0.001$), and EE ($P < 0.001$) and for 2 h after exercise ($P < 0.001$). There were no significant differences between groups at any individual time point.

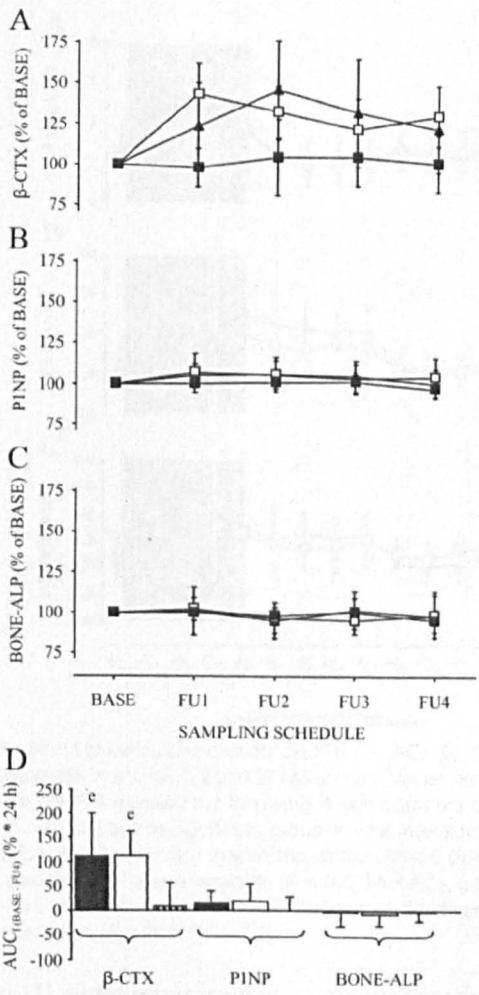

FIG. 3. The percent change in BASE concentrations of β-CTX (A), P1NP (B) and bone ALP (C) on the four recovery days (FU1–FU4) after exhaustive exercise in the RA (□), ET (▲) and CON (■) groups. Values are mean ± 1 SD. LMM ANOVA revealed the following: a significant main effect of time ($P < 0.001$) and a significant group × time interaction ($P < 0.01$) for β-CTX and no significant effect of exercise for P1NP (group × time interaction, $P = 0.536$) or bone ALP (group × time interaction, $P = 0.397$). D, $AUC_{I(BASE-FU4)}$ analysis of β-CTX, P1NP, and bone ALP in the RA (*white bars*), ET (*black bars*) and CON (*hatched bars*) groups. Values are mean ± 1 SD. One-way ANOVA revealed the following: significant for β-CTX ($P < 0.01$) and not significant for P1NP ($P = 0.414$) or bone ALP ($P = 0.970$). e, Different ($P < 0.01$) from CON.

PO_4 was significantly increased from BASE during FD ($P < 0.001$), at EE ($P < 0.001$), and for 2 h after exercise ($P < 0.05$) in both groups (Fig. 5C) but was similar to BASE on the four follow-up days. There were no significant differences between groups.

Discussion

Exhaustive running was associated with a sustained increase in bone resorption, as assessed by β-CTX, in RA and ET men. The 45% increase in β-CTX is similar to that reported by Guillemant *et al.* (10) after 60 min of cycling at 80% VO_{2max} but smaller than the 75% increase re-

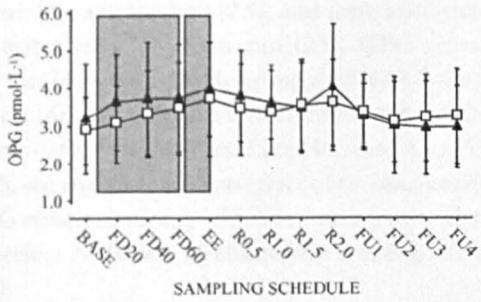

FIG. 4. Effect of exhaustive exercise on OPG concentrations in the RA (□) and ET (▲) groups. Values are mean ± 1 SD. LMM ANOVA revealed the following: a significant main effect of time ($P < 0.001$) but no significant group × time interaction ($P = 0.082$).

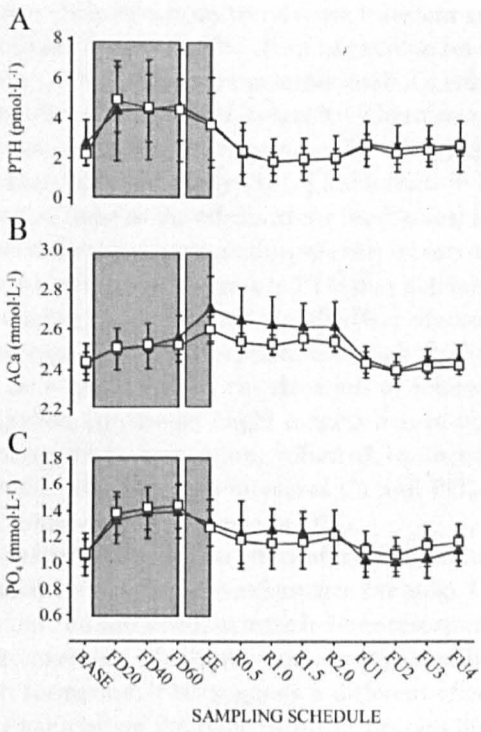

FIG. 5. Effect of exhaustive exercise on PTH (A), ACa (B), and PO_4 (C) concentrations in the RA (□) and ET (▲) groups. Values are mean ± 1 SD. LMM ANOVA revealed the following: a significant main effect of time ($P < 0.001$) but no significant group × time interaction ($P = 0.464$) for PTH, a significant main effect of time ($P < 0.001$) and a significant group × time interaction ($P < 0.01$) for ACa, and a significant main effect of time ($P < 0.001$) but no significant group × time interaction ($P < 0.398$) for PO_4.

ported by Kerschan-Schindl *et al.* (6) immediately after a 246-km running race.

Smaller increases (10–20%) after 50 min of cycling (11) or no increase after a 60-min run (19) have also been reported. Herrmann *et al.* (9) reported a significant increase in β-CTX at 3 and 24 h after 60 min cycling at 110% of anaerobic threshold, although they did not standardize the time of day or the nutritional status of subjects before sample collection. Kerschan-Schindl *et al.* (6) report a 40% increase in β-CTX 3 d after the start of a 246-km run but did not control the nutritional status of subjects before sample collection. Thus, ours is the first controlled study to show that β-CTX is elevated in response to exhaustive running and remains so for over 24 h after exercise.

Bone formation, as indicated by P1NP and bone ALP concentrations, was not altered by exhaustive exercise. Previous studies report no change in P1NP immediately after 30 min of treadmill walking (20) or one-legged knee extensor exercise (21) and decreased P1NP concentrations at 3 and 24 h after 60 min of cycling (9). Our study is the first to measure P1NP beyond 24 h of recovery and suggests no sustained effect of acute exercise on bone formation up to 96 h. The follow-up period in our study was limited to 4 d, and it is possible that P1NP might have increased beyond this point. Studies report increased C-terminal propeptides of procollagen type 1 between 24 and 96 h after exercise (7, 8, 22). The lack of changes in bone ALP agrees with previous findings (10) and is consistent with bone ALP being involved in the continuous mineralization of bone rather than early mineralization.

Increased bone resorption, without a concomitant increase in formation reflects an alteration to the bone remodeling balance. This has been described previously (10), but ours is the first study to report this effect beyond 2 h after exercise in a controlled laboratory setting. Bone resorption plays a role in stress fracture development, where it transiently weakens bone, making it vulnerable to further mechanical stresses and fracture (4). The high incidence of stress fracture injury reported in military recruits (1) might result from increases in osteoclastic activity associated with repeated bouts of exhaustive exercise. Applicability to the athletic population may be limited because the exercise protocol was not reflective of typical athletic activity. However, because the intensity, duration, and exhaustive nature of the exercise are common elements of athletic training, our results might partly explain why studies report stress fractures (2) and low BMD (3) in athletes.

This is the first controlled study to examine the influence of training status on the responses of bone resorption and formation markers to acute exhaustive exercise. There was no significant difference in basal bone metabolism between our groups suggesting that the bone remodeling balance remains unchanged by endurance training. Previous studies report higher concentrations of OC (13) and bone turnover (23) in trained individuals. However, Brahm *et al.* (24) reported a lower rate of bone turnover in regular exercisers compared with their less active counterparts.

This is the first study to examine the time course of changes in OPG with acute exercise and showed that OPG increased after only 20 min of exercise and was maintained up to the morning after exercise. Previous studies have shown increased OPG concentrations immediately after a

246-km run (6), a marathon (25), and resistance exercise (26) but not after a 15.8-km run (25). OPG remained increased for 24 h in our study compared with 3 d after a 246-km run (6), which might reflect differences in the exercise stimuli of the two studies. Like Herrmann and Herrmann (27), we also observed no effect of training status on basal OPG concentrations, which is consistent with there being no effect of 10 wk of endurance training on basal OPG (28).

Increases in circulating OPG in conditions associated with significant bone loss have been interpreted to indicate a compensatory response, albeit partially ineffective, to increased bone resorption (29). We observed increased β-CTX and OPG, which might reflect a compensatory effect. Although the temporal association of changes in β-CTX and OPG with exercise is not known, OPG only remained increased for up to 24 h. It is possible that the initial increase in OPG matched that of RANK-L but subsequently diminished, allowing excess RANK-L to stimulate osteoclasts. Serum RANK-L has previously be shown to decrease immediately after a marathon (25) but increase 3 d after the start of a 246-km run (6). We did not measure RANK-L because previous studies have shown that it is undetectable in about 55% of postmenopausal women (30) and about 70% of healthy controls (31). As such, it is possible that the inclusion of RANK-L data using current assays or the expression of the OPG to RANK-L ratio might be misleading. However, we acknowledge that without a measure of RANK-L using a suitably sensitive assay, any interpretation of the association between OPG and bone turnover is incomplete.

The PTH response to acute exercise was unaffected by training status, with concentrations increased during exercise but returning to BASE within 30 min of recovery. Nishiyama *et al.* (13) showed no effect of training status on changes in PTH with acute exercise in young men, although others have shown that the PTH response in older men is enhanced after training (32).

In the present study, ACa was increased with exercise, whereas increases in lactate were modest, suggesting that a reduction in calcium or the presence of metabolic acidosis did not increase PTH. The increase in PTH may have contributed to the increase in ACa by stimulating calcium reabsorption in the kidney and then by osteoclastic resorption of bone (33). Changes in serum PO_4 precede changes in PTH (34), whereas acute exogenous PO_4 results in increased PTH (35). These findings suggest that the increase in PO_4 may have increased PTH with subsequent osteoclastic bone resorption contributing to the sustained increase in PO_4.

When given exogenously, PTH has dual effects on human bone (36). Prolonged infusions increase bone resorption, whereas daily injections (producing transient spikes in PTH) induce formation. The effect of exercise on PTH was transient, which suggests a possible anabolic effect in bone. Anabolic effects of PTH in rats have been reported with peak concentrations in the same order of magnitude as those observed in our study (37). The increase in PTH might sensitize bone to the effects of the mechanical loading (38), with the rapid increase during exercise serving to maximize this effect. The increase in PTH may activate the bone remodeling cycle, but the overall effect of exercise was an increase in bone resorption, although this interpretation may be limited by the duration of follow-up. Taken together, our results might suggest that increased PTH increases bone resorption, reflected by increased β-CTX levels, which in turn increases Ca and PO_4 and induces a compensatory increase in OPG.

In conclusion, there was no effect of training status on the bone turnover response to exhaustive running. There was a marked and sustained increase in bone resorption in response to exercise, which was not accompanied by an increase in formation. This suggests a different effect of exhaustive exercise on the bone turnover process during recovery, which might explain previous findings of deleterious effects on the skeleton in those who partake in regular endurance exercise. The increase in serum OPG might represent a compensatory response to the increase in bone resorption or could simply be a direct effect of exercise. The increase in PTH may be responsible for the increased bone resorption and cannot be explained by decreased calcium but might be related to the increase in circulating PO_4.

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Address all correspondence and requests for reprints to: Jonathan P. R. Scott, Room G077, Building A54, QinetiQ, Cody Technology Park, Ively Road, Farnborough, Hampshire GU14 0LX, United Kingdom. E-mail: jscott1@qinetiq.com.

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