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The role of supplementary calcium in submaximal exercise and endurance performance

By

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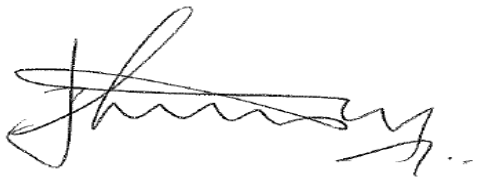

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ABSTRACT

Epidemiological data suggest a positive relationship between increased calcium intake and decreased fat and total body mass in healthy people (McCarron et al. 1984, Davies et al., 2000). An *in-vitro* model suggesting the role of cyclic Adenosine Monophosphate (cAMP) and phosphodiesterase 3B (PDE 3B) has been implicated in the relationship between calcium and lipolysis (Xue et al., 2001). The objective of the series of studies presented in this thesis was to investigate the ergogenicity of supplementary calcium in endurance exercise via studying its influence on substrate metabolism and body composition.

Study 1 examined the effects of four weeks of calcium (citrate) supplementation (1000 mg elemental calcium/day) on 60 minutes of cycling at a submaximal intensity of 50%$W_{peak.}$ The results of this study indicated that calcium supplementation significantly improved body composition of the participants with a greater fat loss and increased lean mass observed in highly trained athletes as compared to the recreationally trained participants. In addition, four weeks of calcium supplementation also showed an enhanced trend of availability of fatty substrates in the plasma and consequently an increased trend towards higher fat oxidation during submaximal exercise.

Study 2 and 3 thus examined the effects of calcium supplementation directly on performance during endurance events (25 and 10 mile cycling time trials (TT)) in highly trained athletes. Results from these studies indicate that following calcium

supplementation there was a meaningful improvement in power output (PO) (~4%) during the 25TT and ~2.7% in 10TT with corresponding better completion times in both the time trials. This amounts to an increase in ~2-3% higher $PO_{\%peak}$ and ~3-5% increase in power output at lactate threshold, with the higher percentages attributable to the 25TT and the lower end to the 10TT. This increase in PO was achieved without the corresponding increase in oxygen consumption, thus significantly improving the gross efficiency of the cyclists. In addition, four weeks of calcium supplementation once again exhibited the same trends in body composition as observed in study 1 of decreased fat mass and a concurrent increase in lean mass.

The results from this set of investigation indicate that calcium plays a multifactoral role in performance enhancement of endurance events. This may have been achieved via a combination of influence on substrate metabolism and body composition. The impact of calcium on fatigue mechanisms and vascular tone may have also played a part in increasing PO and thus efficiency of the athletes. Thus the potential of calcium to enhance performance needs further investigation in studies with larger sample sizes and different training status of athletes.

LIST OF PUBLICATIONS

BOOK CHAPTERS

- Jawadwala, R. (2011) Dietary Calcium – A potential ergogenic aid? Book chapter in Duncan M. J. (Ed.) *Trends in Human Performance Research*. New York: Nova Science Publishers. ISBN 9781616685911

CONFERENCE PROCEEDINGS

- Jawadwala, R., Atkins, S. and Lowe, N. (2012) Effect of four weeks of calcium supplementation on plasma non-esterified fatty acids during a twenty five mile cycling time trial. To be published in *Journal of Physiology (Biomedical Basis of Elite Performance Conference, London, UK)*

- Jawadwala, R., Atkins, S., Lowe, N. and Robinson, P. (2011) Effect of calcium supplementation on cycling performance in 10 mile time trials. *FASEB J,* 25:608.1 *(Experimental Biology Annual Conference, Washington DC, USA)*

- Jawadwala, R., Atkins, S., Lowe, N. and Robinson, P. (2011) Effect of calcium supplementation on body composition of highly trained athletes. *FASEB J,* 25:354.3 *(Experimental Biology Annual Conference, Washington DC, USA)*

- Jawadwala, R., Atkins, S., Lowe, N. and Robinson, P. (2010) Effect of calcium supplementation on cycling performance in 25 mile time trials. *Journal of Sports sciences (BASES Annual Conference, Glasgow, UK)*

- Jawadwala, R., Atkins, S., and Lowe, N. (2009) Effect of calcium supplementation on body composition and total energy expenditure during submaximal exercise. *Medicine and Science in Sports and Exercise*, 41: 5 *(ACSM Annual Conference, Seattle, USA)*

- Jawadwala, R., Atkins, S., and Lowe, N. (2008) Effect of calcium supplementation on substrate metabolism during submaximal exercise. *Proceedings of the Nutrition Society*, 67: E371 *(Nutrition Society Annual Conference, Nottingham, UK)*

- Jawadwala, R., Atkins, S., and Lowe, N. (2008) Is Calcium Ergogenic? Faculty of Science and Technology Annual Research Conference. *Scientific Proceedings, University of Central Lancashire.* (Prize winner) *(UClan Postgraduate Research Day, Preston, UK)*

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Last, but certainly not forgotten, Rees. Thank you for the rhymes, that lovely addiction to Earl Grey tea (coffee still rules though) and continually reminding me of that exclamation mark after “The End”.

LIST OF ABBREVIATIONS

Adenosine monophosphate	AMP
Adenosine triphosphate	ATP
Aerobic lactate threshold	$LT_{aerobic}$
American college of sports medicine	ACSM
AMP activated protein kinase	AMPK
British Cycling Federation	BCF
Calcium	Ca^{2+}
Calciferol	D_3
Carnitine palmitoyl transferase	CPT
Central nervous system	CNS
Coefficient of variance	CV
Coenzyme A	CoA
Cohen's effect size	*d*
Confidence intervals	CI
Coronary artery risk development in young adults	CARDIA
Cyclic adenosine monophosphate	cAMP
Delta efficiency	DE

Extensor digitorum longus	EDL
Energy expenditure	EE
Energy intake	EI
Estimated Average Requirement	EAR
Ethylenediaminetetraacetic acid	EDTA
External work	EW
Fat free mass	FFM
Fat mass	FM
Fatty acids	NEFA
Free extracellular calcium	$_{o}[Ca^{2+}]$
Free intracellular calcium	$_{i}[Ca^{2+}]$
Food Standards Agency	FSA
Glucose oxidase	GOD
Glycerol kinase	GK
Glycerol phosphate oxidase	GPO
Gross efficiency	GE
Half life	*t½*
Heart rate	HR
High dairy	HD

Hormone sensitive lipase	HSL
Intact parathyroid hormone	iPTH
International Units	IU
Intra muscular triacylglycerol	IMTG
Lactate threshold	LT
Limits of Agreement	LoA
Low dairy	LD
Low density lipoprotein	LDL
Maximal oxygen consumption	$\dot{V}O_{2max}$
Megajoules	MJ
Messenger ribonucleic acid	mRNA
Microlitres	µl
Millilitres	ml
Millimetres	mm
Millimolar	mmol
Minute/s	min
Nanometre	nm
National Health and Nutrition examination survey	NHANES
National Diet and Nutrition Survey	NDNS

National Institute of Health	NIH
Non esterified fatty acids	NEFA
Nuclear vitamin D receptor	nVDR
Parathyroid hormone	PTH
Peak heart rate	HR_{peak}
Peak oxygen consumption	$\dot{V}O_{2peak}$
Peak power output	PO_{peak}
Pearson's correlation coefficient	r
Pearson's product moment correlation coefficient	R^2
Percentage of peak power output	$PO_{\%peak}$
Peroxidase	POD
Personal computer	PC
Phosphodiesterase 3B	PDE 3B
Physical Activity Readiness – Questionnaire	PAR-Q
Plasma membrane calcium-ATPase	PMCA
Potassium	K^+
Power output	PO
Power output at lactate threshold	PO_{LT}
Probability value	p

Rate of appearance	R_a
Rating of Perceived Exertion	RPE
Recommended Dietary Allowance	RDA
Recommended Nutrient Intake	RNI
Respiratory exchange ratio	RER
Resting metabolic rate	RMR
Revolutions per minute	rpm
Ten mile TT	10TT
Twenty five mile TT	25TT
Sarco/endoplasmic reticulum calcium-ATPase	SERCA
Schoberer Rad Messtechnik	SRM
Specific glucose transporters	GLUT
Standard deviation	SD
Standard temperature, pressure and dry	STPD
Thermic effect of meal	TEM
Total energy expenditure	TEE
Triacylglycerol	TAG
Training Impulse Score	TRIMP
Typical error	TE

Uncoupling proteins	UCP
Viable yellow agouti mutant mice	A^{vy}
Volume of carbon dioxide consumption	$\dot{V}CO_2$
Volume of oxygen consumption	$\dot{V}O_2$
Work efficiency	WE
1, 25, Dihydroxy Vitamin D_3 (calcitriol)	$1,25(OH)_2D_3$
25 Hydroxy Vitamin D	25(OH)D

CHAPTER 1

INTRODUCTION

"On his way back to Japan in 1960, he passed through Britain and took the opportunity of coming to Cambridge to visit Alan Hodgkin and myself. He told us of his observations, and also said that Lipmann had been unwilling to accept that anything as simple as a calcium ion could perform such an important and specific function, and that this had delayed Ebashi's publication of his results. Later, Ebashi told me that he had been much encouraged by my enthusiastic response to what he told us".

(Huxley, 2007)

The identification of factors that influence energy balance is an important issue in the research field of nutrition. Specific sport populations are concerned with energy balance from a number of perspectives. Maintaining ideal body mass/composition for competitions, making weight in certain sports are some of the primary concerns for athletes. Apart from genetic differences, energy intake and physical activity levels as well as the macro-nutrient composition of the diet are generally considered some of the major factors that explain variations in energy balance. However, recent

experimental evidence has emphasized that a positive or negative energy balance may happen via the influence of factors that have no *a priori* calorific value. This is the case for calcium intake. Gruchow et al. (1988) analysed a survey database (NHANES1) in a study designed to investigate the relationship between diet and hypertension. An inverse relationship between dietary calcium and body weight was subsequently reported. Following that, data that established a mechanistic link between calcium intake and energy/fat balance were reported (Zemel et al., 2001). Based on these epidemiological and *in-vitro* studies there has been a flux in intervention trials in the recent years to establish a link between dietary calcium via dairy and/or supplemental route and energy balance. Most of the studies have looked at different dosages, experimental conditions and outcome measures with mixed results. Part of the confounding issue lies in the fact that it is generally accepted that the uncertainty associated with optimal calcium intakes and requirements exists primarily because the body is able to homeostatically adapt to various levels of dietary intake. Thus, it does not seem that the *in-vitro* data can lend itself in a straightforward manner to more physiological dose responses of dietary calcium *in-vivo*. Most of the studies in this area have been conducted on overweight and obese population, or if they have been conducted on 'normal weight' populations it has generally been sedentary participants with or without recreational exercise. For highly trained athletes these studies do not lend to a representative population from which they can make use of the data for multiple reasons. First of all being trained, athletes have well adapted metabolically regulated pathways for optimal energy utilisation specific to their training (Noakes, 2000, Holloszy and Booth, 1976). Secondly, the physiological mechanisms governing energy balance over a long

period of time, which apply to overweight and obese populations, may differ in the athletic population (Bloom et al., 1976).

Fat oxidation is an important substrate in exercise that lasts for more than 20 min (Romijn et al., 1993). Therefore if calcium supplementation improves lipolysis it may be an important area for consideration in endurance event. Moreover, the effect of calcium on body composition might also be important to endurance athletes where power-to-weight ratios play a defining role in endurance performance.

Therefore, the purpose of this investigation was to evaluate the role of supplementary calcium in submaximal exercise and endurance performance in a non-calcium deficient population. Two outcomes were hypothesised through which these links may enhance performance; one, via changes in fuel metabolism and second via reduced body fat and/or increased lean mass.

Thus, the aims of this thesis are to assess the effects of calcium supplementation on

1. Substrate metabolism during submaximal exercise.
2. Body composition changes in athletes.
3. Endurance performance.

Accomplishment of these aims should enable a greater understanding of the role of calcium in endurance performance via changes in fat metabolism during exercise aswell as its longitudinal effects on body composition of the athletes.

These aims will be accomplished by means of the following objectives –

1. To determine the effect of four weeks of calcium supplementation on changes in fat metabolism as well as total energy expenditure during a submaximal cycle ergometry test exercise.
2. To examine the affect of the training status of the athlete on the putative metabolic changes due to calcium supplementation.
3. To examine the effect of calcium supplementation on changes in body mass and composition of athletes during a four week period.
4. To assess the effect of calcium supplementation on endurance cycling performance indicators such as time to completion, power output and gross efficiency.
5. To identify the optimal range of endurance events that may benefit from the ergogenic effects of calcium.

Achievement of these objectives requires at first an exploratory experiment to study the metabolic changes in a broad range of athletic population, ranging from recreational to well-trained. Further experiments designed to study performance indicators in endurance events of differing duration are required.

1.1 Organisation of the thesis

The thesis begins with the review of the current and relevant literature (Chapter 2) providing a background summarising the research that has lead to our current knowledge of the relationship between dietary calcium and energy balance, proposed mechanistic links, and the potential merits of calcium as an ergogenic aid in certain

sporting conditions. Following this, a section on the general methodology and instrumentation (Chapter 3) used in all the experiments during the investigation are described and includes suitability, validity, reliability and sensitivity of the measures. Chapter 4 is a report on the experiment conducted to study shifts in substrate metabolism due to calcium supplementation during steady state submaximal exercise (50% W_{peak}) as well as changes in body composition over four weeks in a mixed gender heterogeneous sample of recreational and well-trained athletes. Based on the results in Chapter 4, further experiments were designed to highlight the ergogenic role of calcium in sport performance with a more homogenous sample that showed a greater response to the intervention in Chapter 4. Thus, Chapter 5 reports these effects in a moderate intensity event (25 mile cycling time trial) and Chapter 6 reports the effects of calcium supplementation on a more intense endurance event (10 mile cycling time trial) using highly trained cycling time trialists. Chapter 7 provides a synthesis of the findings along with the limitations encountered during the process of investigation, whilst, Chapter 8 outlines the inferences drawn from the investigations and identifies further directions for research.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

This chapter reviews the current research regarding calcium and its role in metabolism. It highlights the role of calcium in the cellular processes that regulate fuel utilisation with the help of studies conducted on *in-vitro*, animal and human models. The review also aims to concurrently describe the metabolic changes that happen as a consequence of exercise and the impact of duration and intensity of exercise on substrate metabolism. Finally, it consolidates the role of calcium in substrate metabolism in exercise with relation to due changes in energy balance and body composition studies.

2.2 Dietary calcium

Milk and dairy foods are the main sources of calcium in the British diet, contributing to 27 to 51% of the total calcium intake (Finch et al., 1998). However the contribution from cereals is also significant as these products (many of which are fortified with calcium) are consumed in relatively large amounts. Table 2.1 provides

the calcium content of a range of commonly consumed foods. There is no marked difference between ages or gender in proportion of calcium intake accounted for by different food groups. The contribution of dietary supplements to calcium intake is negligible apart from the oldest age group of adult women (50-64 years) who increased their mean daily intake by 10% with supplements (Henderson et al., 2003).

Table 2.1 Calcium content of commonly consumed foods (mg/100g)

Food	Calcium content mg/100g
Cheddar cheese	720
Sardines (canned in oil)	550
Milk, skimmed/semi-skimmed	120
Milk, whole	115
Bread, white	110
Eggs, boiled	57
Bread, wholemeal	54
Apples	4

Source: Manual of Nutrition (Food Standards Agency (FSA), 2008)

Table 2.2 presents the Reference Nutrient Intakes (RNI) for calcium for males and females within different age groups (Henderson et al., 2003). These are considered sufficient to meet the nutritional requirements of 97.5% of the population. According to the trends in British national dietary surveys, average dietary calcium appears to exceed this and may even be increasing. In the United States (US) for example, the Recommended Dietary Allowances (RDA) for calcium differ slightly: 1000 mg/d for

children aged 4-8; 1000 mg/d for adults aged 19-50 (both men and women); and 1200 mg/d for female adults over 50 (Ross et al., 2010). Furthermore the trend in calcium intake appears the same in the US and Canada (Ross et al., 2010).

Table 2.2 Recommended Nutrient Intakes for calcium (mg/d)

Age		RNI (mg/d)
Children	Up to 12 months	525
	1-3 years	350
	4-6 years	450
	7-10 years	550
Males	11-18 years	1000
	19+ years	700
Females	11-18 years	800
	19+ years	700
	Pregnancy	1000
	Lactating	+ 550

Source: COMA-Committee on Medical Aspects of Food Policy (1991)

Data from the National Diet and Nutrition Survey (NDNS) for adults aged 19 to 64 years indicate that the mean intake of calcium is 1007 mg/d (144% of the RNI) for men and 777 mg/d (111% of the RNI) for women. However, in young women and teenage girls, intakes are generally < 400 mg/d (Smithers et al., 2000, Henderson et al., 2003). As peak bone mass is achieved during late adolescence/early adulthood, a low intake of calcium during periods of growth could have implications for bone

health in later years (for example, by increasing the risk of osteoporosis) (Theobald, 2005a).

Calcium intakes are an important consideration for athletic population, since athletes are exposed to physiological stressors above and beyond those of sedentary individuals. Petrie et al., (2004) have reported an increased need for vitamins and minerals in general in athletes due to decreased gastrointestinal absorption, increased sweat losses, losses incurred via urine and faeces, and increased needs associated with tissue maintenance and repair. However, it has been shown that exercise and the elevated metabolism associated with exercise itself does not increase the body's need for dietary calcium. However electrolytes are often lost in high amounts due to sweat concentration and so dietary calcium intake may need to be augmented to compensate for those losses and to prevent deficits from occurring (Petrie et al., 2004). As such there are no increased RNI suggested for athletes, except for amennorheic athletes where 1500 mg/d is recommended in the US (Kunstel, 2005)

2.3 Role of calcium in the body

Calcium is quantitatively the most abundant mineral of the body and a vital electrolyte. Besides structural support, calcium is required for critical biological functions such as nerve depolarisation (conduction), muscle contraction, cell adhesiveness, mitosis, and blood coagulation (Alberts et al., 2004). An average adult body contains about 1.5 kg of calcium of which is 99% is in the skeleton (Dickerson, 2003).

Calcium in blood maybe protein bound, complexed and ionised, or free. The ionised fraction is the focus of metabolic control, especially through parathyroid hormones and vitamin D, and is kept constant at 1.0 – 1.2 mmol/l by processes that continuously add or remove the calcium (Vaskonen, 2003). Para-thyroid hormone (PTH), which is produced by chief cells located in two pairs of parathyroid glands situated adjacent to the thyroid gland, increases plasma calcium concentration through three types of actions :-

- Mobilization of calcium out of the bone by PTH which is released rapidly by stimulation of S nerves in the thyroid glands or circulating catecholamines, and thus stimulation calcium mobilization from the bone after acting on osteoclasts for several hours (Abe and Sherwood, 1972).
- More rapid mobilisation of calcium out of osteoid or recently formed bone, mediated by osteocytes in the presence of Vitamin D and without resorption of mineralised bone (Borer, 2003).
- PTH increase of calcium reabsorption in distal tubule of the kidney by PTH and stimulation of synthesis of Vitamin D_3 in the proximal tubule (Friedman et al., 1996).

During hypocalcaemia, the decreased calcium is sensed in the parathyroid glands by the calcium sensing receptor (Borer, 2003). PTH secretion is increased and acts to restore calcium to normal by its effects on bone and kidney. By stimulating renal 1-hydroxylase it accelerates the conversion of 25-hydroxy vitamin D (25(OH)D) to calcitriol ($1,25(OH)_2D_3$); the latter acts on intestinal calcium absorption in conjunction with PTH on bone and kidney to restore calcium levels to normal (Borer, 2003). PTH secretion is returned to normal by restoration of normocalcemia

and also by direct suppressive and anti-proliferative effects of calcitriol on parathyroid glands. Vitamin D receptors are present in the parathyroid glands and act as sensors for the detection of adequate levels of calcitriol, thus regulating PTH synthesis and release (Schulkin, 2001).

2.3.1 Calcium distribution and tissue concentrations

For an organism to carry out its varied functions and even to survive the calcium concentration in biological fluids must be maintained within narrow limits. The total plasma calcium concentration (2.15 to 2.55 mmol/l) which normally fluctuates only by ± 3% - is maintained by a number of homeostatic mechanisms, most notably PTH, vitamin D and thyrocalcitonin (Bowers and Rains, 1988). These three substances regulate the calcium concentration as a resultant of three actions: net absorption from the gut, net loss through the urine and net deposition in the bone.

Approximately 99% of the total body calcium is present in the bone, which acts as a reservoir to maintain the requisite supply of calcium to the tissues via the circulation. The remaining 1% in the circulation is not completely biologically active since 50% of the plasma calcium is bound to plasma proteins. Plasma ionised calcium concentrations range from 1.0 to 1.2 mmol/l in healthy adults (Robertson et al., 1981). The calcium concentrations of most tissues tend to cluster around the value of 1µ mole/gram wet weight. This is true for muscle (skeletal, cardiac and smooth) as well as peripheral nerve and brain (Bowers Jr and Rains, 1988).

Most of the calcium in a cell exists in the bound state. It is bound to plasma and cellular membrane components, to collagen and mucopolysaccharides, and other

negatively charged constituents of cells (Alberts et al., 2004). The basis for this avid binding may, at least in part be ascribed to the interaction between calcium and acidic phospholipids (Hauser and Dawson, 1968).

While exact quantification of each component of cell calcium has proved quite difficult to measure, it is even more difficult to discern a shift in calcium from one cell compartment to another. Experiments which have employed radioactive calcium (^{45}C) to localise different cell fractions have shown them to be multi compartmentalised and kinetically definable into different cell fractions (Alberts et al., 2004). There is a fraction that is readily interchangeable with the extracellular medium with a half-life (*t½*) of 1-5 min, a less readily exchangeable fraction with a *t½* of about 30 min, and a non-exchangeable fraction (Blaustein, 1974). However, that fraction may represent a summation of efflux from more than one compartment, which makes it difficult to relate these fractions to a specific cellular entity (Blaustein, 1974).

2.3.2 Role of calcium as a second messenger

The role of calcium as an intracellular second messenger is based on its regulation at a nanomolar level. While the extracellular calcium concentration is about 1.2 to 2.0 mmol/l, $_{i}[Ca^{2+}]$ is usually about 100 to 200 nmol/l (Rasmussen, 1971). In most cells large calcium gradients exists across the intracellular membranes of calcium storage organelles such as the endoplasmic and sarcoplasmic reticulum. $_{i}[Ca^{2+}]$ stores do not have a homogeneous structure and cannot be attributed to a single cellular organelle (Rasmussen, 1971). In order for calcium to exert its effects, it must gain access to critical intracellular sites but the plasma membrane limits the

entry of calcium. In most excitable cells the resting permeability to calcium is quite low (Rasmussen, 1971). But during contraction this may transiently increase many folds. It is the molecular diversity of the associated proteins in calcium signalling that determine the specific interactions between stimulus and change in the state of the organelle. Since most of the cellular calcium exists in the bound state, it seems reasonable to contemplate that at least certain substances initiate secretion by acting on calcium storing structures to release bound calcium. There is evidence to indicate that stimulation of secretory process in some glands may be effected by altering the state of bound cellular calcium (Rasmussen, 1971). This appears to be the situation in regard to the action of pituitary hormones on the adrenal cortex (Borer, 2003) and thyroid gland (Alberts et al., 2004). Whether the critical calcium fraction is extracellular or intracellular, translocation of this cation is a fundamental requirement for many physiological processes.

In skeletal muscle four important apparatus have been identified that play an important role during contraction and relaxation. The ryanodine receptor that is the sarcoplasmic reticulum Ca^{2+} release channel, the troponin protein complex that mediates the Ca^{2+} effect to the myofibrillar structures leading to contraction, the Ca^{2+} pump responsible for Ca^{2+} reuptake into the sarcoplasmic reticulum, and calsequestrin, the Ca^{2+} storage protein in the sarcoplasmic reticulum (Alberts et al., 2004). Different calcium gradients are established by active transport mechanisms. The calcium pumps are located either in the plasma membrane (plasma membrane calcium-ATPases, PMCAs) or in the membrane of ${}_i[Ca^{2+}]$ stores (as sarco/endoplasmic reticulum calcium-ATPases, SERCAs) (Carafoli and Brini, 2000). For both types of calcium pumps, various isoforms have been described that differ in their activation and regulation properties.

Upon electrical stimulation of intact muscle fibres, cellular calcium increases because of a release of calcium from intracellular stores or through a calcium entry across the plasma membrane, or both. The sequence of these processes depends on the cell type and type of stimulation (Carafoli and Brini, 2000). The amount of calcium inside the stores is limited. Therefore calcium release from the stores is only a transient signal. However, for a cellular activation, often a sustained change of intracellular signal is needed, making a calcium entry via plasma membrane calcium channels necessary (Berridge et al., 2000). When the surface membrane and transverse tubular are depolarised it leads to the release of calcium from the sarcoplasmic reticulum via ryanodine receptors (RyR), this elevates cytosolic calcium levels to ~100 fold higher. The calcium then binds to one of the troponin sub-units on the filament, leading to muscle contraction (Fig. 2.1).

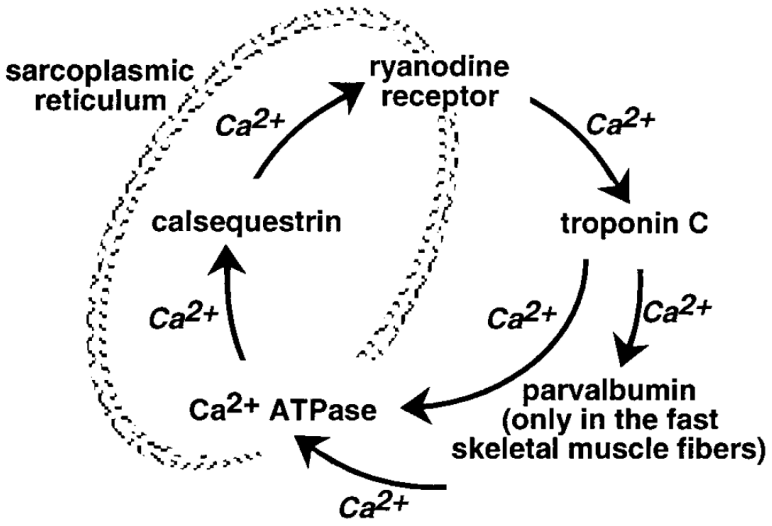


Fig. 2.1 **A schematic representation of the role of calcium in initiating muscle contraction (Berchtold et al., 2000)**

Different types of calcium channels have been described with regard to their structure and their mode of activation: voltage dependant, stretch activated, receptor operated and store operated (Alberts et al., 2004). The complex nature of cellular calcium regulation and signalling is responsible for temporally and spatially distinct calcium signals such as calcium transients and for plateaus as well as calcium oscillations and calcium waves (Berridge et al., 2000). There is now growing evidence that these different calcium signalling patterns encode for different cellular effector functions (Clapham, 2007).

The amount of calcium taken up during contraction may be related to the extracellular calcium concentration and enhanced influx may not be maintained during prolonged stimulation but may decrease with time (Bianchi and Shanes, 1959). The inactivation of the calcium current prevents the intracellular calcium concentration from increasing to a point where it would be deleterious to the cell and may, at least in part, account for the decrease in the response commonly observed during prolonged stimulation of intact fibres in *in-vitro* experiments (Bianchi and Shanes, 1959). If the Ca^{2+} control mechanisms cannot compensate for the release, the excess of cytoplasmic Ca^{2+} causes sustained muscle contraction and rigidity, increased energy production and consumption, as well as ADP, CO_2, and heat production. Finally, the activation of degradative enzymes leads to membrane damage and cell death (Fig. 2.2).

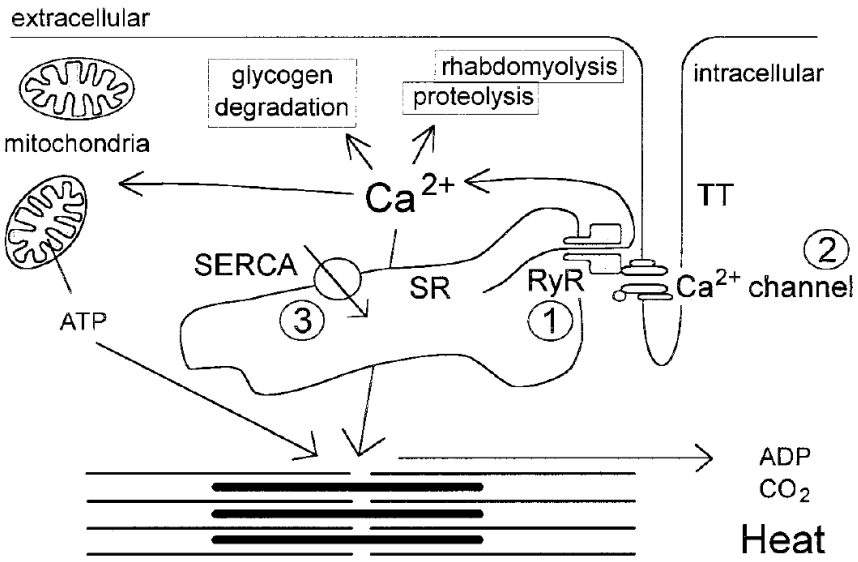


Fig. 2.2 A schematic representation of the effect of sustained increased intracellular calcium is implicated in various metabolic disturbances. 1. Structural changes in RyR leads to 2. opening of the calcium channels, which then cause the 3. activation of SERCA. Malfunctioning at any of the points may lead to long term metabolic disturbances (Berchtold et al. 2000)

Thus, Changes in $_{i}[Ca^{2+}]$ concentrations can evoke a number of cellular responses including proliferation, motility, oxidative burst, secretion of cytokines and gene expression and are involved in the pathogenesis of several diseases such as inflammatory diseases (Resnick, 1999), cardiovascular diseases (Resnick, 1999) and neurological disorders (Greenberg, 1997).

2.4 Regulation of substrate metabolism in exercise

2.4.1 Regulation of fat metabolism during exercise

Lipid fuel sources are important energy substrates for skeletal muscle metabolism during endurance exercise. Their contribution to total oxidative metabolism is dependent on a variety of factors, including exercise intensity and duration as well as dietary and training status. Oxidizable lipid fuels include circulating plasma triacylglycerols (TAG) and non-esterified fatty acids (NEFA), as well as intramuscular triacylglycerols (IMTG). However the specific rate of oxidation is determined by energy requirements of working muscles, non-esterified fatty acids (NEFA) delivery to muscle mitochondria and the oxidation of other substances (Achten and Jeukendrup, 2004).

After an overnight fast most energy needs at rest are provided by oxidising NEFA derived from adipose tissue triacylglycerols. At rest the amount of NEFA released from AT typically exceeds the amount oxidised (Coppack et al., 1994); such that the rate of NEFA appearance in the plasma (R_a) is approximately twice the rate of NEFA oxidation. Therefore large portions of NEFA liberated by lipolysis of adipose tissue triacylglycerols are re-esterified back into TAG, principally by the liver (Ranallo and Rhodes, 1998). However, during exercise NEFA do not account for more than 60% of the maximal rate of oxygen consumption (Klein et al., 1994). A high concomitant glucose oxidation is required for high intensity endurance exercise and power output falls when glycogen stores are exhausted. It is not clear whether this limited rate of oxidation of NEFA represents a limitation in the rate at which they can be delivered to and transported in the plasma (Klein et al., 1994, Sidossis et al., 1998) or in the rate in which they can be taken up and oxidised by the muscle

(Wolfe et al., 1990). Fig 2.3 shows the maximal contribution of energy expenditure derived from glucose and NEFA, taken up from the blood and minimal contribution of muscle triglyceride and glycogen stores after 30min of exercise, expressed as function of exercise intensity (Romijn et al., 1993).

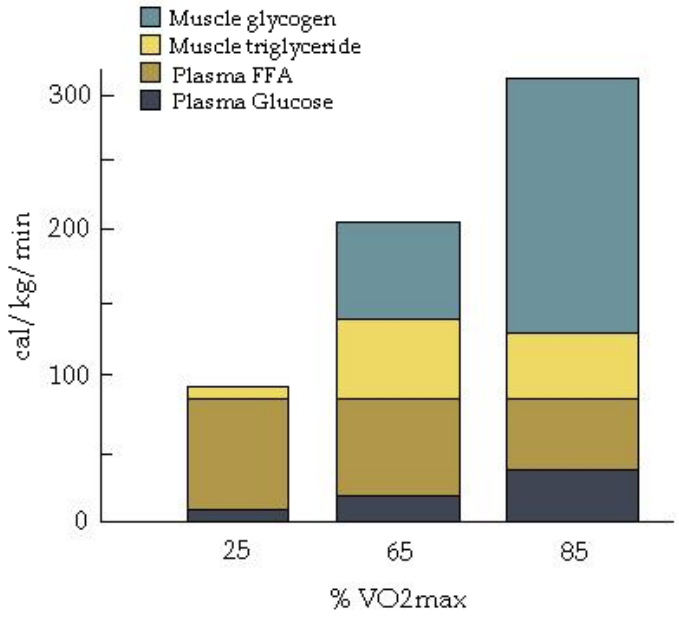


Fig.2.3 Maximal contribution of different substrates towards energy expenditure after 30mins of exercise at different intensities. Adapted from Romijn et al. (1993)

To obtain the energy stored in the NEFA they must be sequentially oxidised. Firstly NEFA must be released from the TAG stored within the adipocytes which are stimulated by hormones such as adrenaline during both exercise and in the fasting state (Horowitz and Klein, 2000a). NEFA released when TAG molecules are hydrolysed has two major fates, re-esterification to a TAG following attachment of Coenzyme A (CoA) to the NEFA and leaving the adipocyte, as a NEFA in the blood stream. If all the NEFAs released during lipolysis exit the adipocyte then there should be a NEFA to glycerol ratio of 3:1 in the blood (Galbo et al., 1975, Klein et

al., 1994). However, because of the reutilization of some of the NEFAs in the formation of new TAG molecules, this 3:1 ratio is seldom observed. Indeed, during inactivity nearly two thirds of the NEFAs produced during lipolysis are re-esterified (Coppack et al., 1999). On the other hand, during light to moderate intensity exercise most NEFAs produced by lipolysis leave the adipocyte (Hodgetts et al., 1991). Thus neurohumoral stimulation or inhibition is the determining factor for the extent of lipolysis.

Plasma NEFA turnover is also highly dependent on the recent dietary intake. After an overnight fast, fat supplies virtually all the energy demands of the body at rest in healthy individuals, with a plasma NEFA turnover rate of 4-5 µmol/kg/min being sufficient to meet this requirement (Horowitz and Klein, 2000a). Peripheral lipolysis is highest during low intensity exercise (25%$\dot{V}O_{2max}$) (Watt et al., 2002, Hawley, 2002) and does not increase further with more intense exercise (at 65-85%$\dot{V}O_{2max}$) (Hawley et al., 2000, Sidossis et al., 1997). However, total fat oxidation is about 40% higher at 65%$\dot{V}O_{2max}$ compared with exercise at 25%$\dot{V}O_{2max}$ (Romijn et al., 1993). The implication of this being that at higher work intensities there is a greater contribution of IMTG to total fat oxidation. Both IMTG and plasma NEFA provide equally to fat oxidation at 65%$\dot{V}O_{2max}$. There are several explanations to this such as, uptake of plasma NEFA by the exercising muscle is less than the measured rate of fat oxidation (Klein et al., 1994). Aas well as a decline in , IMTG concentration (55-75%$\dot{V}O_{2max}$) (Bergman and Brooks, 1999).

At higher work intensities (85%$\dot{V}O_{2max}$) most of the required energy is derived from carbohydrate sources (Romijn et al., 1993). This is a result of the recruitment shift from type I and type IIa to type IIb muscle fibres for this intensity of work, and fast

twitch muscle fibres do not have the oxidative capacities of slow twitch fibres (Hawley et al., 2000). Whole body lipolysis of peripheral adipose tissues does not decrease during high intensity exercise (85%$\dot{V}O_{2max}$) this is demonstrated by the R_a of glycerol which remains high throughout the exercise period (Klein et al., 1996). One reason for the decreased oxidation of NEFA could be explained by an entrapment of NEFA in the adipose tissue during exercise with a release after exercise. Bülow and Madsen (1976) have reported large increases in adipose tissue blood flow (ATBF) during exercise (Fig. 2.4) in response to the β-adrenergic stimulation. As seen in the graph, even though the ATBF increases multiple fold during prolonged exercise, it does not match the systemic NEFA concentration increase.

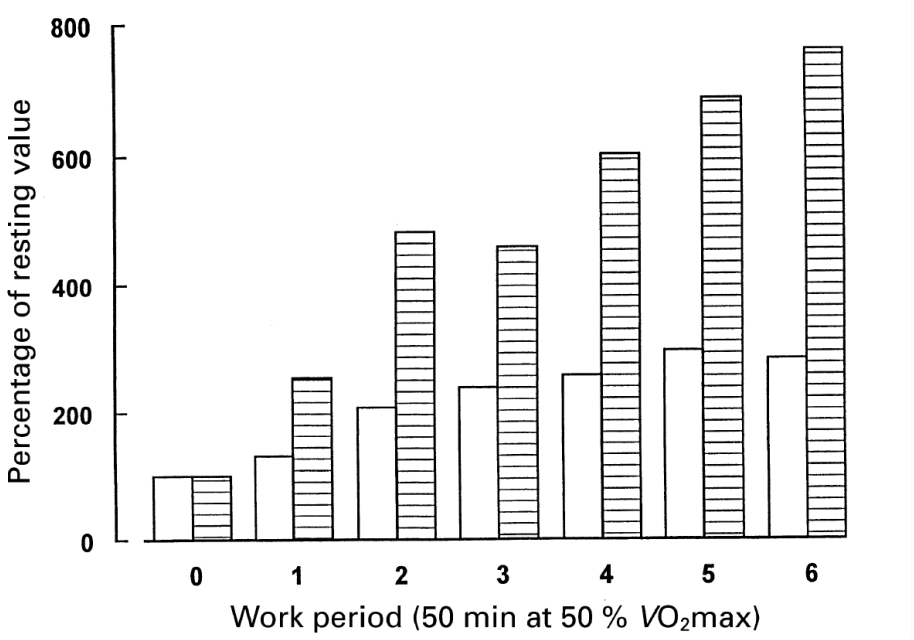


Fig. 2.4 Relative changes in ATBF (white bars) and systemic NEFA concentrations (lined bars) during prolonged exercise (Bülow and Madsen, 1976)

Thus the plasma NEFA concentration increases dramatically during the minutes immediately after high intensity exercise, despite a simultaneous reduction in lipolysis (decreased rate of R_a of glycerol) (Klein et al., 1996). Also the hydrophobic molecules of NEFA can be limited in their transport in the blood due to the limited binding capacity of albumin (Ranallo and Rhodes, 1998). At higher intensities of exercise there is a higher concentration of lactate in the blood. In contrast to the understanding that lactate inhibits lipolysis, it is possible that lactate probably increases re-esterification of NEFA into adipose tissue triglycerides (Dyck et al., 1993). A reduction in the blood flow to the adipose tissue can also reduce the release of NEFA during high intensity exercise (Wolfe et al., 1990).

During moderate intensity exercise, in the range of 55% to 75% of $\dot{V}O_{2max}$, that can be maintained for 60 minutes or longer, there is a progressive decline in the proportion of energy derived from muscle glycogen and muscle triglycerides, and a progressive increase in plasma NEFA oxidation (Gollnick et al., 1981, Ranallo and Rhodes, 1998, Romijn et al., 1993). During the first 30 minutes or so of moderate intensity exercise in the fasting state, plasma NEFAs and IMTG provide roughly equal amounts of the fat that is oxidized. When the exercise is continued beyond 30 minutes, oxidation of plasma NEFAs provides progressively more of the total energy requirements, compensating for the decreased utilization not only of IMTG but also of muscle glycogen (Sidossis et al., 1997) (Fig. 2.5). This increase in plasma NEFA oxidation during prolonged exercise is made possible by a progressive rise in plasma NEFA concentration, and is necessitated by a progressive depletion of muscle glycogen and triglyceride stores (Romijn et al., 1993).

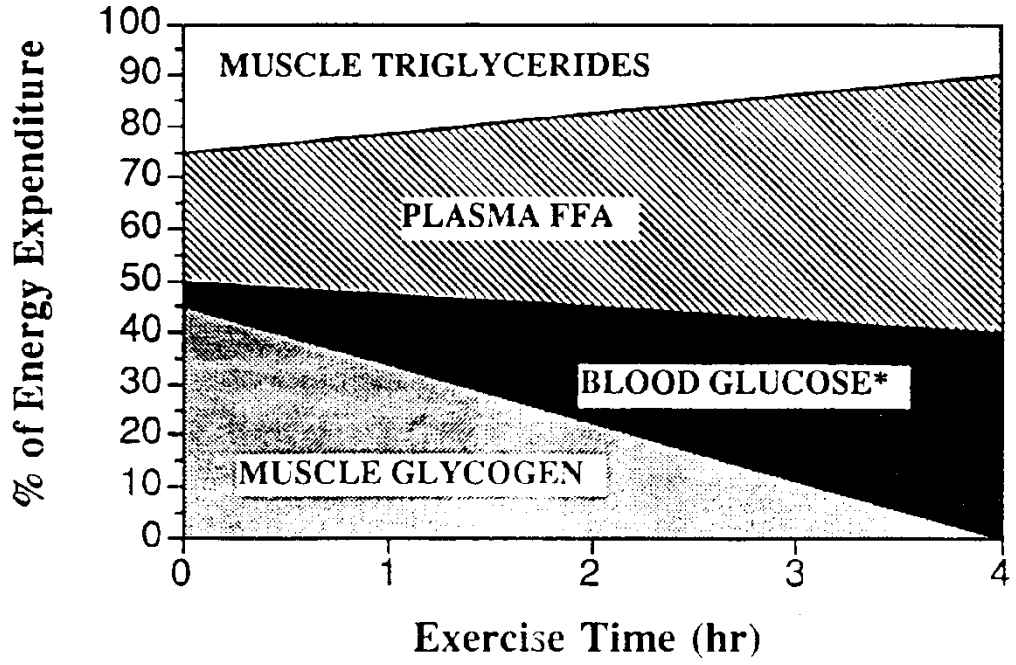


Fig. 2.5 Relative contribution of blood borne and intramuscular substrates to energy production during 120 min of exercise at 65% $\dot{V}O_{2max}$ (Romijn et al., 1993)

It has been suggested that IMTG is an important energy source for skeletal muscle metabolism, especially during prolonged exercise. These suggestions are based on the fact that isotopically determined plasma NEFA oxidation does not match estimates of total lipid oxidation as calculated from respiratory exchange ratios (RER) measurements during prolonged exercise (Klein et al., 1994). In post absorptive exercising men the oxidation of circulating plasma NEFA can account for little more than half of the total lipid oxidation (Sahlin et al., 1990). These results have led researchers to suggest that NEFA released either from intramuscular TAG pool or from adipose tissue located between muscles fibres are utilised for oxidative metabolism in exercising muscles. At 25%$\dot{V}O_{2max}$ muscle TAG breakdown occurs to a minimum (< 10%) as the availability of NEFA derived from the plasma is adequate to meet the substrate requirements for that intensity of exercise (Sahlin et al., 1990, Wolfe et al., 1990, Romijn et al., 1993). At increasing intensities (65%$\dot{V}O_{2max}$)

muscle TAG supplies about 50% of the total fat metabolism during the first 60mins of cycle ergometry. However with an increase in exercise duration at the same intensity muscle TAG could only supply about 30% of the total fat metabolised after 120 min of exercise (Romijn et al., 1993, Ranallo and Rhodes, 1998). These results support the notion that the breakdown of IMTG occurs to compensate for the sluggish response of NEFA release at the onset of moderate exercise. The more type I fibres involved in the exercise the greater the depletion of TAG not stored in the muscle fibres. With increasing recruitment of type IIa fibres during moderate to heavy exercise there is an increase in depletion of IMTG (Horowitz and Klein, 2000a). IMTG utilisation could also be influenced by the mode of exercise, bicycle and heavy resistance exercise are associated with marked increases in the plasma catecholamine levels, whereas one legged knee extension exercise is associated with catecholamine levels that are barely above resting concentrations. This lower catecholamine response could possibly affect the utilisation of IMTG since it has been shown in exercising humans that non selective β-adrenergic blockade prevents muscle TAG lipolysis (van Loon, 2004).

Among other factors that affect substrate metabolism the rate of lipolysis is highly dependent on the actions of several hormones which in majority include the catecholamine – epinephrine and norepinephrine and insulin. The other hormones that exert their influence on the rate of stimulation of lipolysis are – growth hormone, adrenocorticotropic hormone, thyroid stimulating hormone and parathyroid hormones (Ranallo and Rhodes, 1998). Because only catecholamines can effectively stimulate lipolysis at physiological concentrations, they appear to be the major important stimulators of lipolysis in the human adipose tissue *in-vivo*. In human adipose tissue, catecholamines have both ά-adrenergic inhibitory and β-

adrenergic stimulatory effects on the rate of lipolysis via corresponding changes in the activity of adenlyl cyclate and in the intracellular production of cyclic adenosine monophosphate (cAMP) (Hodgetts et al., 1991, Galbo et al., 1975).

Insulin is another hormone which regulates lipolysis. The action of insulin is opposite to that of the catecholamines, in that it is antilipolytic. Thus insulin prevents lipolysis via a sympatho adrenally mediated alpha adrenergic stimulation (Bergman and Brooks, 1999). During exercise of long duration, the insulin level is either unchanged or slightly decreased although lipolysis progressively increases. Thus the insulin level is unlikely to be the controlling mechanism for the rate of lipolysis (Wolfe et al., 1990). It is possible that the decrease in insulin levels in the early stages of exercise has a permissive effect that facilitates an amplification of lipolysis in the later stages (Horowitz and Klein, 2000a).

2.4.2 Integration of fuels during prolonged exercise

It is well established that a mixture of carbohydrates and lipids are used as fuels in skeletal muscle at rest and during exercise (Wolfe et al., 1990, Romijn et al., 1993, Watt et al., 2002, Sahlin et al., 1990). Studies using Respiratory Exchange Ratio (RER) demonstrates that both carbohydrates and lipids are used during exercise and that their relative contribution changes as a function of exercise intensity and duration and pre exercise diet (Romijn et al., 1993, Romijn et al., 1995). Low power outputs are associated with greater oxidation of lipids, while higher exercise intensities result in greater reliance on carbohydrates. The release of NEFAs into the blood from adipose tissue stores rises in parallel with exercise intensity to approximately 50% of $\dot{V}O_{2max}$, and then gradually declines. On the other hand

release of glucose into the blood from the liver increases with exercise intensity. (Brooks, 1997) has proposed a crossover concept to explain the fuel utilisation during exercise in terms of the balance between carbohydrate and fat (Fig.2.6). The crossover point is that relative exercise intensity where adenosine tri-phosphate (ATP) formation from the use of carbohydrate exceeds that of lipid. Exercise at power outputs beyond this point will rely more and more on carbohydrate oxidation and less and less on fat (Brooks, 1997).

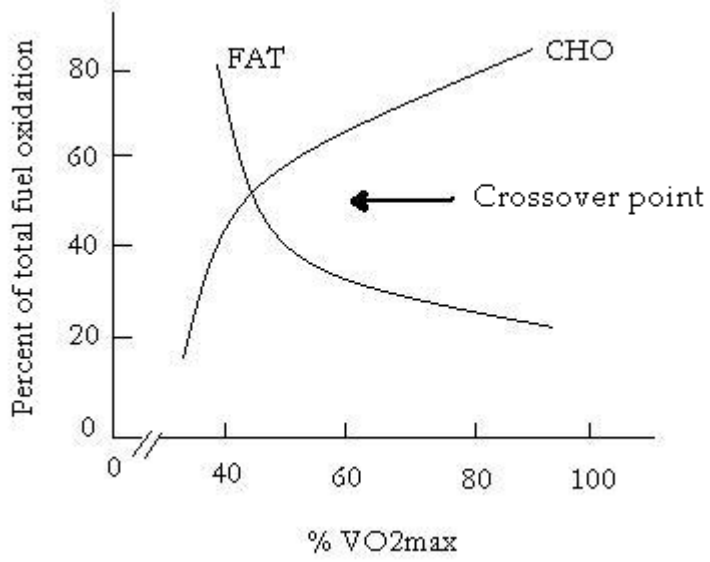


Fig.2.6 Crossover point concept as proposed by Brooks (1997)

There are several sites in the pathways of carbohydrates and lipid metabolism where fuel selection during exercise may be regulated. These include regulation at sites controlling carbohydrate metabolism and oxidation as in the glucose-fatty acid cycle (Randle et al., 1963). The relationship between glucose and NEFA metabolism is reciprocal and not dependant. The essential mechanism involves an allosteric control which inhibits CHO oxidation prior to conversion to pyruvate dehydrogenase. An

increase in plasma NEFA levels increases uptake and oxidation of fatty acids. This leads to an increase in the acetyl CoA to CoA ratio in the mitochondria. Increase in citrate due to β-oxidation in the TCA cycle leads to leakage of citrate from mitochondria into the cytosol. Here citrate binds to phosphofructokinase decreasing the rate of glycolysis (Fig. 2.7).

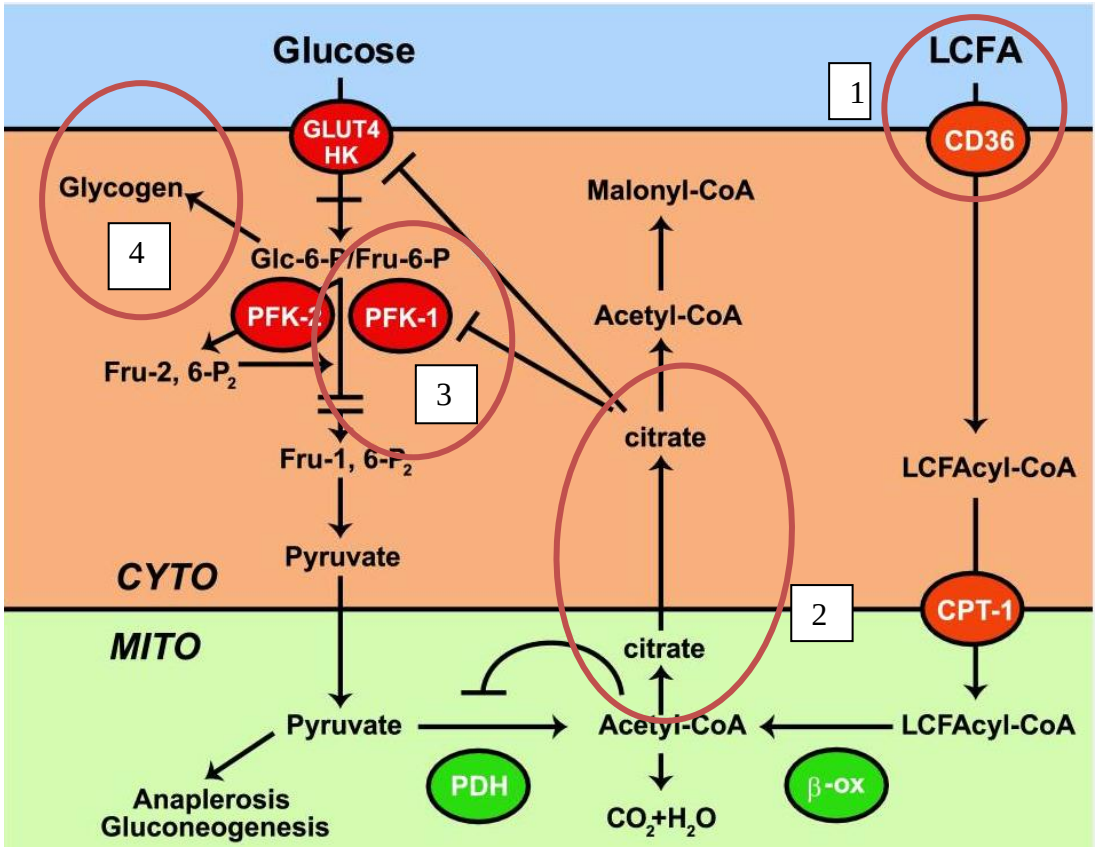


Fig. 2.7 A schematic representation of the glucose-fatty acids cycle. Increase in long chain fatty acid (LCFA) oxidation leads to increase acetyl CoA/CoA ratio. This leads to citrate accumulation in the cytosol, inhibiting phosphofructokinase-1 (PFK-1) and thus reducing glycolysis. Adapted from Hue and Taegtmeyer (2009)

An alternative hypothesis to the classic Glucose-fatty acid cycle is that malonyl CoA concentrations can reflect the availability of carbohydrates as a substrate (i.e. fuel sensing) with increased glycolytic flux producing more pyruvate and thus

increasing acetyl CoA/CoA and malonyl CoA, thus reducing NEFA oxidation by reducing transport of long chain NEFAs into the mitochondria via inhibition of carnitine palmitoyl transferase (CPT) (McGarry et al., 1977) (Fig. 2.8).

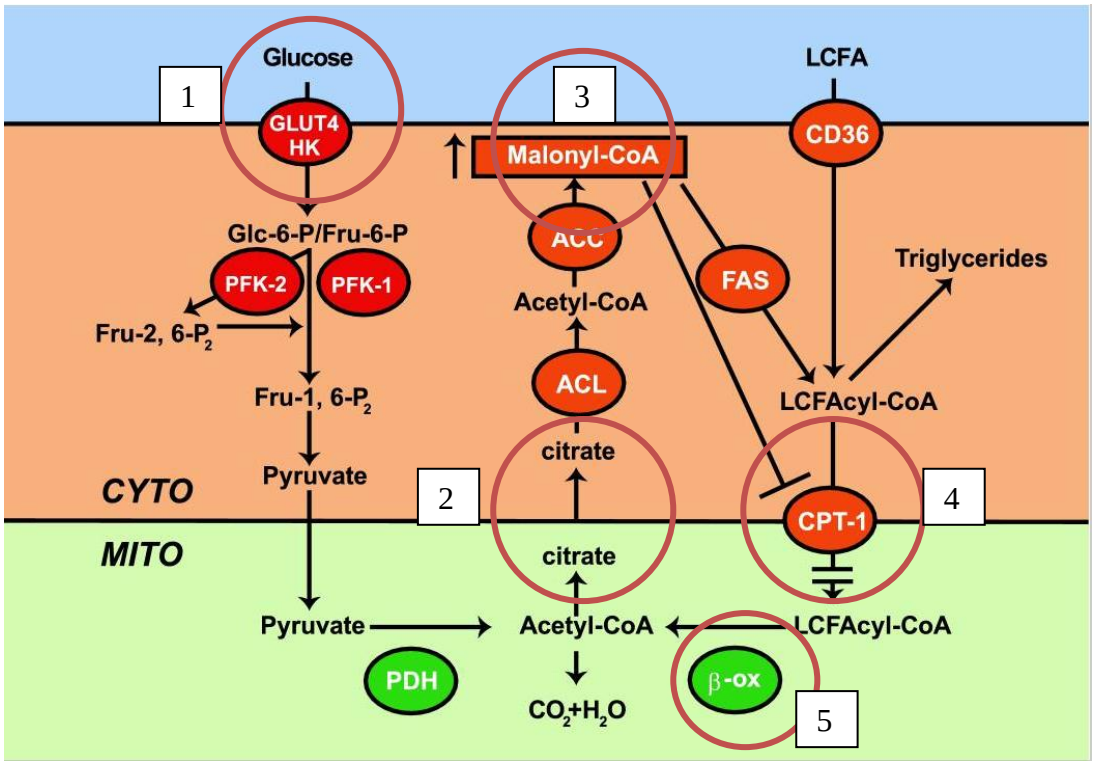


Fig 2.8 A schematic representation the regulation of fat oxidation via carbohydrates. Increased glycogenolysis increases acetyl CoA/CoA ratio, leading to citrate accumulation in the mitochondria. This leads to increase in malonyl CoA in the cytoplasm where it inhibits CPT-1 leading to decreased transport of fatty acids into the mitochondria. Adapted from Hue and Taegtmeyer, (2009)

Under hemodynamic stress conditions, the inhibition of carbohydrate oxidation by fatty acids is markedly reduced leading to an activation of AMP-activated protein kinases (AMPK) (Goodwin and Taegtmeyer, 1999). AMPK is also activated during exercise and physical activity (Vavvas et al., 1997). Changes in $_{i}[Ca^{2+}]$ can also activate AMPK independently (Kahn et al., 2005). Unlike the situation in liver, where malonyl-CoA can be actively converted into fatty acids, muscle contains very

low levels of fatty acid synthase (FAS). Accordingly, in muscle cells, malonyl-CoA is thought to act as a "fuel sensor" whose primary role is to regulate the rate of fatty acid oxidation. Furthermore, its concentration appears to be controlled by the relative activity of acetyl-CoA carboxylase-2 (ACC-2) and malonyl-CoA decarboxylase (MCD), both of which are regulated by AMP-activated kinase (AMPK) (Andersson et al., 2004) (Fig. 2. 9).

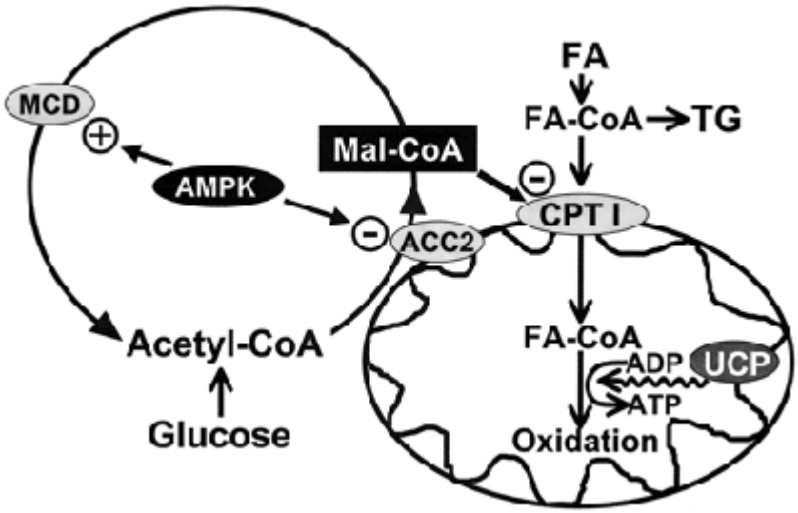


Fig. 2.9 A schematic representation of the interaction of glucose and fatty acid metabolism in muscle (Andersson et al., 2004)

Also, an increasing body of evidence is now suggesting the role of malonyl CoA in the regulation of energy balance and thus obesity (Ruderman et al., 2003). The possibility that malonyl CoA and AMPK sense fuel availability in the hypothalamus where they play a role in initiating signalling events that regulate food intake is an important consideration in fuel metabolism (Ruderman et al., 2003)

Therefore, in conclusion, during submaximal exercise lipolysis and the release of NEFA from the adipocytes exceeds uptake by the peripheral tissues and the net

result is an increase in plasma NEFA. As the intensity of exercise increases, lipolysis declines with a relative decrease of fat contribution and a concurrent increase in glucose metabolism. At workloads above 65%$\dot{V}O_{2max}$, the limiting factor for prolonged exercise appears to be the glycogen stores. Thus regulation of the fuel substrates during exercise is dependent on the intensity and duration of the exercise, in addition to the training status, mode, dietary status and hormonal regulation.

2.5 Putative mechanistic theories of the role of calcium in energy and substrate metabolism

It has been shown that a low calcium intake tends to be a marker for a poor diet generally (Barger-Lux et al., 1992). Historically, primitive human diets were calcium rich; with calcium to energy ratios two to four times that of modern humans (Eaton and Nelson, 1991). The hormonal response to a low calcium intake is an increase in calcitropic hormones such PTH and a corresponding increase in 1, 25, Dihydroxy cholecalciferol ($1,25(OH)_2D_3$) concentrations. Because a low calcium intake would have been tantamount to a low food intake, it may be that the PTH and $1,25(OH)_2D_3$ response evoked by a low calcium intake to regulate its energy metabolism and thereby adapt to imminent food shortage (Davies et al., 2000). Today, with the calcium intake disconnected from energy intake, the primitive energy conserving response with a low calcium intake may predispose to weight gain.

The framework for understanding this 'anti-obesity' effect of dietary calcium derives from studies of the mechanism of action of *agouti*, the first obesity gene to be cloned. Obese *agouti* mutant mice (viable yellow, A^{vy}) exhibit increased $_i[Ca^{2+}]$ in

several tissues (Zemel et al., 1995). The increase in $_i[Ca^{2+}]$ is closely correlated with both the degree of ectopic *agouti* expression and body weight. This correlation has led to several mechanistic links between *agouti*, $_i[Ca^{2+}]$ and regulatory enzymes in lipid metabolism. In the forefront of this research is the work of Zemel and colleagues who demonstrated that *agouti* protein stimulates calcium influx and promotes energy storage in human adipocytes by inhibiting lipolysis and stimulating *de novo* lipogenesis (Zemel et al., 2000). When dietary calcium is limited, plasma calcium levels fall, stimulating the secretion of PTH, which in addition to increasing calcium release from bone through increased bone resorption and reduced renal calcium loss, also promotes the hydroxylation of 25 Hydroxy Vitamin D ($25(OH)_2D$) to its active form, $1,25(OH)_2D_3$. Both PTH and $1,25(OH)_2D_3$ are implicated in the increase of $_i[Ca^{2+}]$ (Xue et al., 1998). This has lead to the hypothesis that calcitropic hormones may play a role in adipocyte fat metabolism.

Xue et al. (2001) reported the link between increased $_i[Ca^{2+}]$ and lipogenesis in rat adipose tissue. They suggested that the anti-lipolytic effect of $_i[Ca^{2+}]$ may be mediated by the activation of phosphodiesterase 3B (PDE-3B) leading to a decrease in cyclic adenosine monophosphate (cAMP) and phosphorylation of HSL and consequently inhibition of lipolysis (Fig. 2.10).

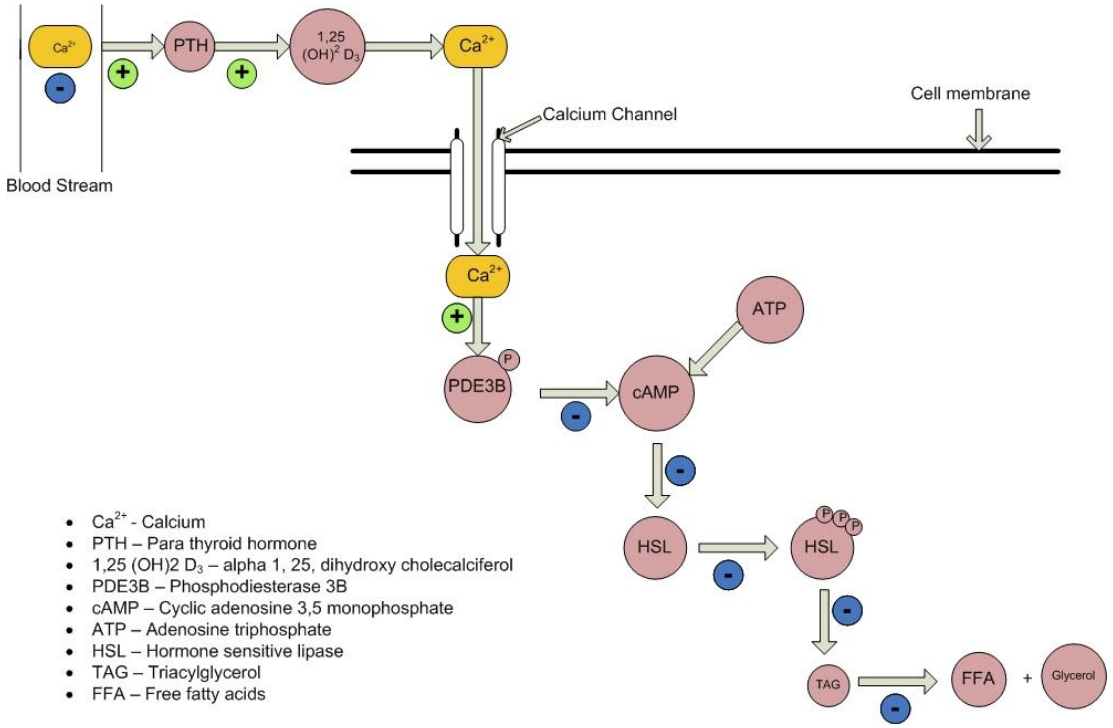


Fig. 2.10 Schematic representation of the role of calcium in fat metabolism *in-vitro*

In addition to the anti-lipolytic role of $1,25(OH)_2D_3$ it may also be involved in the regulation of thermogenesis and energy metabolism in humans. $1,25(OH)_2D_3$ acts via a nuclear Vitamin D receptor (nVDR) to inhibit the expression of uncoupling protein 2 (UCP2) (Shi et al., 2001b). UCPs are mitochondrial transporters present in the inner membrane of mitochondria. They have been shown to stimulate mitochondrial proton leak and therefore exhibit a potential role in thermogenesis and energy metabolism (Schrauwen and Hesselink, 2002). Suppression of $1,25(OH)_2D_3$ by feeding high calcium diets to mice, resulting in increased adipose tissue UCP2 and skeletal muscle UCP3 expression and thereby attenuating the decline in core temperature that would otherwise occur on energy restriction has been reported (Zemel and Sun, 2008). In addition physiological concentrations of $1,25(OH)_2D_3$

seem to have an anti-apoptopic effect on adipocytes, mediated by suppression of UCP2 (Zemel and Sun, 2008) (Fig. 2.11).

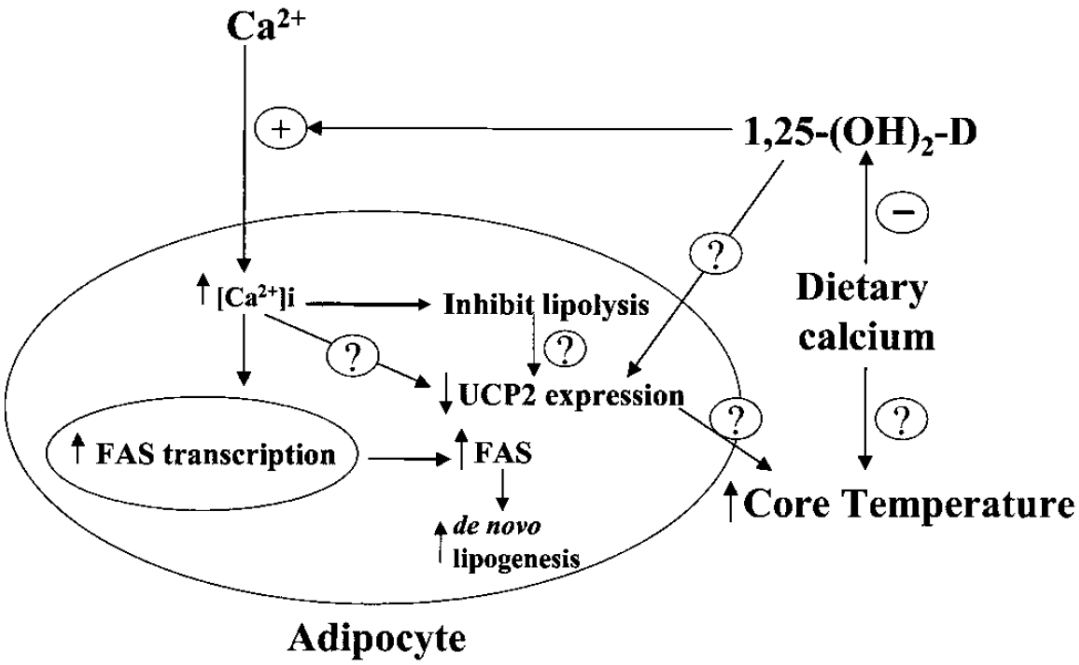


Fig. 2.11 Schematic representation highlighting the possible mechanistic routes in which dietary calcium plays a role on energy metabolism via the influence on UCPs in energy restricted ap2-agouti transgenic mice. Adapted from Shi et al. (2001a)

Another possible mechanism by which dietary calcium intake might affect adiposity is its effect on the absorption of triacylglycerol from the gastrointestinal tract because of formation of non-digestible calcium soaps. Denke et al. (1993) reported that calcium fortification (2200 mg elemental calcium/d) increased the percentage of dietary saturated fat excreted in 72 hour faecal collections from 6% to 13% per day. The high calcium diet also significantly reduced total cholesterol by 6%, LDL cholesterol by 13% and apo-lipoprotein B concentrations by 7% when compared to the low calcium diets. In an 85 day dietary intervention trial, Papakonstantinou et al. (2003) fed rats were fed a high calcium diet containing dairy protein or a control diet. Rats on the high calcium diet gained 29% less carcass fat.

Welberg et al. (1994) studied the effects of calcium supplementation on quantitative and qualitative faecal fat excretion in 24 participants. They reported a dose dependant increase in NEFA excretion with supplemental calcium at 0, 2 and 4 g of calcium per day. These studies on faecal fat excretion predict small effects on total body lipid flux. A person consuming a 2500 kcal/d (~10.5 MJ/d) diet containing one third of energy from fat, taking an additional 2 g elemental calcium/d might be expected to excrete an additional 1% of energy from fat per day and would be anticipated to lose ~3010 kcal/year (~12.6 MJ/year) in stool. This amount of lost energy might explain a change in body weight of ~0.4 kg/year (Parikh and Yanovski, 2003). Thus these data suggest that faecal fat excretion may only explain a small proportion of the effect calcium seems to have on body weight changes.

2.5.1 Calcium and fat metabolism

Based on the above mentioned *in-vitro* studies and epidemiological data, the association between calcium intake and 24-hour fat oxidation in young non-obese humans was first examined by Melanson et al. (2003). They examined the association in both self reported habitual calcium intake (mean 1222 ± 116 mg/d) and acute intake (640 ± 44 mg/d), and found a positive and significant correlation between 24-hour fat oxidation and acute calcium intake but not habitual calcium intake. It is interesting to note that in this study that total calcium and not dairy calcium remained significant predictors of 24-hour fat oxidation in backwards stepwise regression models. They suggested the possibility of a difference in the results between habitual and acute calcium intake on the self-reporting method of calcium estimation. These results have been supported by Cummings et al. (2006)

who reported that, regardless of the source, calcium intake acutely stimulated postprandial fat oxidation and there was a lesser suppression of NEFA following calcium rich meals.

It is also possible that some of the effects of calcium on lipid metabolism could be generated rather quickly as $1,25(OH)_2D_3$ has been reported to exert physiological non-genomic effects on calcium metabolism and its putative effects on increasing $_i[Ca^{2+}]$ (Jones et al., 1998). And indeed in a follow up study Melanson et al. (2005) found a ~10% reduction in circulating $1,25(OH)_2D_3$ concentrations but not in plasma calcium, urinary calcium excretion and plasma PTH levels. In a study by Boon et al. (2005) though not as acute as Melanson et al. (2003) have also reported a significant difference in change in plasma $1,25(OH)_2D_3$ concentrations from 175 ± 16 pmol/l to 138 ± 15 pmol/l in a high dairy/high calcium (1259 ± 9 mg/d ca) group intervention, when compared with a change in plasma $1,25(OH)_2D_3$ concentration from 164 ± 13 pmol/l to 198 ± 19 pmol/l in a low dairy/low calcium (349 ± 8 mg/d) group in a three 7 day dietary intervention study. Thus there seems to be a need for further work to study the effects of an acute load of high calcium foods and its effect on fat metabolism via non-genomic actions of $1,25(OH)_2D_3$ or possible other mechanisms.

In terms of long term interventions, Gunther et al. (2005) have studied the effects of chronic high (1000-1400 mg/d) and low (< 800 mg/d) dairy calcium intake for one year on 19 healthy normal weight women on their respiratory exchange ratio (RER) and fat metabolism after a high (> 500 mg) and low dairy calcium (< 100 mg) meal challenge, before and after the intervention. The intervention group had a greater decrease in RER after both the low calcium (-0.20 ± 0.09 and 0.004 ± 0.08,

$p < 0.0001$) and high calcium (-0.12 ± 0.09 and -0.06 ± 0.08, $p < 0.006$) meal challenges. They also reported a corresponding increase in fat oxidation in the intervention group as compared to the controls after both, the low calcium (0.10 ± 0.05 and 0.005 ± 0.04 g/min, $p < 0.0001$) and high calcium (0.06 ± 0.05 and 0.03 ± 0.04 g/min, $p < 0.009$) meal challenges. These results suggest that chronic increased dietary calcium results in the long term adjustment in the ability to oxidise fats and utilise calories, even without high calcium content in the meal. This however contradicts the findings of Melanson et al. (2003) who have reported no significant changes in fat oxidation rates with a high self reported habitual calcium intake (mean 1222 ± 116 mg/d). Gunther et al. (2005) have not looked at plasma $1,25(OH)_2D_3$ concentrations, however they have reported no significant changes in plasma log PTH for the control group (0.18 ± 0.62) as compared to the intervention group (0.07 ± 0.49) after one year of the intervention, nor any significant correlations with fat oxidation even when corrected for changes in body composition. But they have reported a negative partial correlation between the one year change in log PTH and one year change in whole body fat oxidation in response to a high calcium meal but not a low calcium meal. This probably suggests that the homeostatic concentration of PTH suppression may be modified by the chronic intake of calcium.

If the increase in calcium intake is then combined with a caloric restriction then the effects on fat oxidation rates seem to get more pronounced. In a follow-up dairy intervention study by (Melanson et al., 2005) the authors found that under energy balance conditions there was no effect of low-dairy (LD) (~500 mg calcium/d for 7days) or high-dairy (HD) (~1400 mg calcium/d for 7 days) treatment on respiratory quotient (RQ) or 24-hour macronutrient oxidation. However, under energy deficit

(-600 kcal/d) conditions, 24-hour fat oxidation was significantly increased on high dairy diet (HD = 136 ± 13 g/d, LD = 106 ± 7 g/d, $p < 0.05$). One important distinction between the energy balance and the energy deficit interventions was that energy deficit was achieved via a combination of calorie restriction (~100 kcal) and exercise (~400 kcal from an exercise with workload of 70%$\dot{V}O_{2peak}$). Fat oxidation did not differ between HD and LD in energy deficit conditions when the participants were resting, but only increased during the exercise session and during another two 10 min stepping bouts (96, 72, 96 steps/min). The authors have thus suggested that the increase in fat oxidation rates and decrease in RER during the energy deficit trials could be due to exercise rather than calcium. However, the energy balance trials also included the step bouts without any difference in fat oxidation rates between the two dietary conditions. This could lead to a possible effect of increased lipolytic effect via increase in dietary calcium intakes under energy deficit conditions but not energy balance conditions. This increase in availability of NEFA during exercise conditions could then translate to a higher fat oxidation rate but not in resting conditions where the available NEFA in the blood could be re-esterified due to lower energy demands at the time.

Another study on high (~1700-1800 mg/d) and low (~475 mg/d) calcium for 7 days by Jacobsen et al. (2005) found a small but non-significant increase in fat oxidation with a high calcium + normal protein diet (11.5 ± 8 % EI) but not with high calcium + high protein diet (7.9 ± 6 % EI) when compared to low calcium + normal protein diet (7.4 ± 9 % EI). This was matched with a small but non-significant increase in NEFA after the high calcium + normal protein diet (66 ± 212 µmol/l) as compared to a non-significant decrease (-117 ± 235 µmol/l) in participants who were on a low calcium + normal protein diet and the ones on a high calcium + high protein diet

(-17.5 ± 151 µmol/l). However, unlike the study by Melanson et al. (2005) there was no specific exercise provision in this study. Only spontaneous activity was measured via microwave radar detectors and accounted for as part of the 24-hour energy expenditure (EE). Thus considering the NEFA data, in light of the fat oxidation data, it seems to lend itself to the explanation that the increased availability of NEFA with increased calcium intakes could be an important factor in determining the increased rates of fat oxidation without any change to TEE. Boon et al. (2005) with a similar research of randomised crossover design with isocaloric diets of high calcium/high dairy (1259 ± 9 mg/d), high calcium/low dairy (1259 ± 9 mg/d) and low calcium/low dairy (349 ± 8 mg/d) have also reported very modest non significant increase in fat oxidation rates (108 ± 7 g/d) following high dairy/high calcium diets as compared with low dairy/low calcium group (100 ± 6 g/d).

Considering that most studies have reported very modest changes in fat oxidation rates and it seems that the relation between calcium intakes and substrate metabolism may be more interrelated with lipolysis than oxidation of NEFAs at least in sedentary participants over a 24 hour period. This is evident with observed significant changes in circulating $1,25(OH)_2D_3$ concentrations which has a direct impact on increasing $_i[Ca^{2+}]$ levels at least *in-vitro* and in animal studies affecting lipolytic and lipogenic hormonal milieu as reported by numerous studies (Shi et al., 2001b, Xue and Zemel, 2000, Xue et al., 2001). It is possible that these effects may not reflect normal physiological situations. Another reason for these studies to have reported small but not significant changes in fat oxidation may be due to low statistical power in most human intervention studies described above. Most of these studies have reported studying between 7-20 participants. Boon et al. (2005) have stated that from a post hoc power analysis, it was calculated that to reach significant

differences in substrate metabolism with the variances and differences observed between groups, the required number of participants would at least be 43 in their study. Thus the relationship between fat oxidation rates and calcium intakes in humans requires more in-depth examination particularly the impact of lipolytic mechanisms on substrate metabolism *in-vivo* as that might provide some understanding of the role of calcium in its influence on body composition as seen consistently from epidemiological (McCarron et al., 1984, Zemel et al., 2000, Pereira et al., 2002) and meta analytical (Davies et al., 2000) data.

2.5.2 Calcium and glucose metabolism

Since transport of glucose is a rate limiting step in energy metabolism, the effects of calcium homeostasis on glucose transport may have a valuable role to play in understanding the wide ranging effects of $_{i}[Ca^{2+}]$ in substrate metabolism; especially during exercise. As early as 1914 in the Sheffield laboratory at Yale University, scientists were investigating the influence of calcium in glucose metabolism. The researchers injected normal grown rabbits with different doses of epinephrine, to disturb the sugar homeostasis in the blood and then participanted the rabbits with different doses of calcium salts (lactate and chloride). They reported that rabbits with hyperglycaemia, induced by adrenaline in conjunction with calcium salts resulted in increased elimination of 'sugar' in the urine. Also, withdrawal of calcium produced hypoglycaemia (Underhill, 1916). This suggests that calcium plays an important role in regulating blood glucose.

Many of the current molecular concepts regarding the regulation of glucose uptake have been derived from efforts in the insulin signalling field. The influence and

effects of calcium in the role of glucose uptake is not only complex but also surrounded by controversies and disagreements. It is possible that insulin may increase cytosolic calcium by regulating the activity of calcium channels (Zemel et al., 2000, Shi et al., 2000) which in turn may influence lipolysis (Xue et al., 2001). However, the role of calcium in insulin signalling is reported to be subtle, relying on small, localized changes in intracellular concentration rather than large $_{i}[Ca^{2+}]$ changes mediated by influx from extra- and intracellular compartments (Whitehead et al., 2001). Although calcium is required for proper insulin signal transduction, high concentrations of $_{i}[Ca^{2+}]$ diminish insulin sensitivity (Zemel, 1998). Obese and type 2 diabetic participants exhibit elevated $_{i}[Ca^{2+}]$ levels in many cell types, including insulin target tissues (Kim et al., 1996, Zemel, 1998, Zemel, 2001). Thus changes in calcium levels manipulated either through a dietary route or pharmacologically may have an effect on glucose transport via both insulin signalling and perhaps in the longer term even affect insulin sensitivity. A metabolic shift achieved via increased dietary calcium may therefore influence glucose transport during exercise as well and in turn influence substrate utilization during exercise.

The transport of glucose into the skeletal muscle and other tissues is dependent on a family of facilitative transporters (GLUTs) of glucose. Currently, there are five established functional facilitative glucose transporter isoforms (GLUT 1-4 and GLUTX1), with GLUT-5 being a fructose transporter (Alberts et al., 2004). GLUT-4 isoform is the major insulin-responsive transporter that is predominantly restricted to striated muscle and adipose tissue (Alberts et al., 2004). Calcium seems to be involved in glucose transport via the final stages of docking of GLUT-4 storage vesicles with plasma membrane (Whitehead et al., 2001). Under normal resting

conditions, most of the GLUT-4 molecules reside in membrane vesicles inside the muscle cell (Alberts et al., 2004). In response to insulin or muscle contraction, GLUT-4 translocates to the cell membrane where it inserts to increase glucose transport (Watson and Pessin, 2001). The amount of GLUT-4 protein is a primary factor in determining the maximal rate of glucose transport into skeletal muscle (Rose and Richter, 2005). The mechanism behind the movement of GLUT-4 to surface membranes and the subsequent increase in transport by muscle contractions is largely unresolved, but it is likely to occur through intracellular signalling involving Ca^{2+}-calmodulin-dependent protein kinase, 5'-AMP-activated protein kinase (Kurth et al., 1999), and possibly protein kinase C (Kurth et al., 1999, Persechini and Cronk, 1999). Here calcium plays either a permissive role in insulin stimulated glucose transport or even actively drive the glucose in the tissues (Whitehead et al., 2001).

For more than 30 years the transient "spikes" in $_{i}[Ca^{2+}]$ that occur with muscle contractions have been hypothesized to be involved in contraction-stimulated glucose transport. Holloszy and Narahara (1967) proposed the role of increase in $_{i}[Ca^{2+}]$ by demonstrating that the greater the amount of calcium entering a muscle during a contracture, the greater the increase in glucose transport in the muscle. Other studies have sought to resolve the role of calcium by examining Ca^{2+} activated enzymes. Conventional isoforms of protein kinase such as AMP-activated protein kinase (AMPK) are activated in response to increases in cellular calcium concentrations (Richter and Ruderman, 2009). AMPK is a fuel sensing enzyme that is conventionally activated by increased cellular AMP concentrations, for example during exercise (Richter and Ruderman, 2009). Thus the effect of calcium on AMPK

activation may in part play a role in fuel metabolism, especially during exercise (Fig. 2.12)

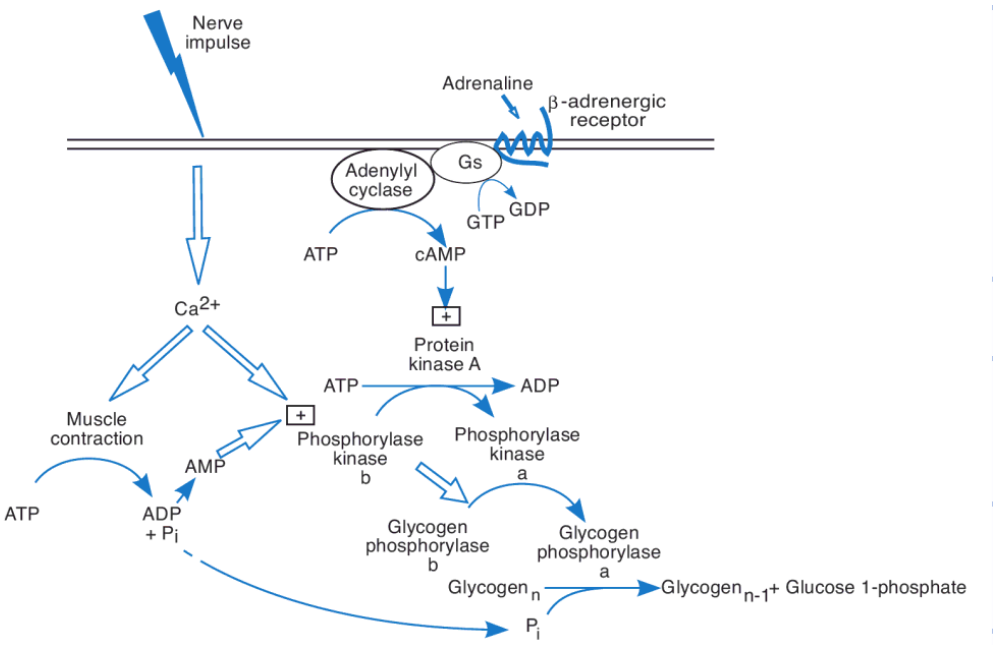


Fig. 2.12 A Schematic representation of the role of calcium in the activation of AMPK

Apart from the above mentioned mechanism of muscle contraction driving glucose transport via a calcium dependant mechanism another perspective involves the role of insulin in increasing free cytoplasmic calcium concentration and this in turn may trigger the stimulation of glucose transport system (Schudt et al., 1976). In their study insulin increased the free cytoplasmic calcium concentration, which may be the intracellular signal for the stimulation of glucose transport. Thus they report that calcium dependency of insulin response could either reflect a calcium controlled transduction of the insulin signal across the plasma membrane or signify that insulin increases $_{i}[Ca^{2+}]$ concentration via enhanced influx from the extra cellular space (Schudt et al., 1976). Thus, these studies indicate that perhaps an increase in dietary calcium may change the extracellular ionic environment which in turn could have

important influences on the uptake of glucose by skeletal muscles during rest and exercise.

2.5.3 Calcium and energy expenditure

To explain the inverse relation between dietary calcium and body weight, a few studies have looked at calcium intakes and its effects on 24 hour energy expenditure (EE) (Jacobsen et al., 2005, Bortolotti et al., 2008, Teegarden et al., 2008). But despite increases in fat oxidation in some studies (Melanson et al., 2005, Boon et al., 2005) there does not seem to be much evidence in relation to increase in Total EE (TEE) with an increase in calcium, intake. Jacobsen et al. (2005) found that calcium intake had no effect on 24 hour EE or fat oxidation after calcium supplementation (1800 mg/d for 1 week). A similar finding by an earlier study by Melanson et al. (2003) found no relation between habitual or acute calcium intakes with 24 hour EE. A recent study by Bortolotti et al. (2008) have also reported no increase in resting EE in overweight or obese individuals on a 800 mg/d supplemental calcium for five weeks. However, Teegarden et al. (2008) reported that overweight women on calorie deficit diets (-500 kcal/d) on supplemental calcium (1200 mg/d) for 12 weeks showed an inverse relationship between PTH and TEE and trunk fat mass. But this relationship was not correlated directly to calcium intakes when compared with controls. However, the same study has reported mean plasma $25(OH)_2$ D to positively correlate with a 12 week change in thermic effect of meal (TEM) (R^2 = 0.17, $p < 0.05$) independent of group assignment. This is in contrast to the findings by Gunther et al. (2005) who have reported a higher TEM after a high

calcium (1000-1400 mg/d) intervention compared with a low calcium (< 800 mg/d) meal challenge, suggesting a metabolic change in TEM mechanisms.

Some studies looking at surrogate measures of calcium metabolism and its effects on EE and resting metabolic rate (RMR) have also not found any effect on energy metabolism with changes in calcitropic hormone levels. Boon et al. (2006) have investigated the effect of 7 day oral supplementation of 2000 IU cholecalciferol on energy and substrate metabolism. Although the oral supplementation in combination with low-calcium (352 ± 31 mg/d) and low cholecalciferol (1.6 ± 0.3 mg/d) diet induced significant changes in plasma $1,25(OH)_2D_3$ concentrations, no differences in RMR or fat oxidations were observed. These are interesting observations in light that tight regulation of blood calcium levels are maintained through hormonal control of PTH and calcitriol and one of the side effects of calcitriol treatment for hypocalcaemia is weight loss (Bortolotti et al., 2008). Many of these studies that have looked at TEE or RMR have also looked at fat oxidation changes with calcium supplementation. Some of them (Boon et al., 2005, Melanson et al., 2005) have reported an increase in fat oxidation levels on the same doses of calcium supplementation perhaps suggestive of a shift in substrate partitioning independent of changes in EE.

2.5.4 Calcium and body composition

Although energy balance is the most critical factor in weight regulation, studies have suggested the calcium supplementation could aid weight loss, in any form of tablet, powder or dairy product. More than 20 years ago a study on the relation between blood pressure and nutrient intake based on data of the 1st National Health and Nutrition Examination Survey (NHANES1) in USA found a significant inverse association between calcium intake and body weight (McCarron et al., 1984). Similar relationships have also been demonstrated in the Australian population, based on an examination of the data from the National Nutrition Survey 1995 (Soares et al., 2004).

A potential relationship between weight loss and calcium supplementation has been noted in animal studies (Papakonstantinou et al., 2003, Tordoff, 2001, Tordoff et al., 1998, Zemel, 2001, Zemel et al., 2000), large epidemiological studies (Pereira et al., 2002) and human intervention trials (Zemel, 2004, Zemel et al., 2005). But, systematic (Trowman et al., 2006) and non systematic reviews (Barr, 2003) have failed to show a definitive statistically significant correlation between calcium supplementation and weight loss. On the other hand Davies et al. (2000) showed a significant negative association between calcium intake and weight for all age groups; on analysing five clinical studies (one intervention and four observational studies). These findings have been confounded by differences in calcium sources, doses, participant demographics, habitual calcium intakes, intervention length and incorporation of exercise and/or energy restriction. Also the imprecision of the methods of estimating habitual (or baseline) calcium and protein intakes renders estimates of the independent variable inherently uncertain. Methods such as seven

day diet diaries and calcium to protein ratios are less prone to errors as compared to the usual food frequency questionnaires.

A recent meta-analysis of data collected over twelve years from a prospective cohort of men in the United States found that total and dietary calcium intakes were associated with lower weight gain in age-adjusted models in the change analysis. But, they failed to reveal a significant association between dietary calcium intake (dairy or no dairy) with long term weight gain with a multivariate model of analysis (Rajpathak et al., 2006). This suggests that other diet and lifestyle factors may explain some of the observed changes in the age-adjusted models. Interestingly, the men with the largest increase in total dairy intake gained slightly more weight (3.14 ± 0.11 kg) than did the men who decreased intake the most (2.86 ± 0.11 kg) ($p < 0.05$). This association was primarily due to an increase in high fat dairy intake. These findings have been supported by other studies; Venti et al. (2005) were unable to find an association between calcium intake and body size ($r = 0.05$) or adiposity ($r = 0.16$) in Pima Indian adults and children. They hypothesised that part of the reason may be their high fat and high energy diet as observed by Rajpathak et al. (2006). These findings from the observational studies have also been substantiated by various controlled randomised trials (Shapses and Riedt, 2006, Thompson et al., 2005, Gunther et al., 2005, Lorenzen et al., 2006) in the recent years.

In addition to the differences in habitual diet related discrepancies, Gunther et al., (2005) found contradictory results from two studies from their own laboratory (Lin et al., 2000) looking at body mass differences with calcium intervention with healthy normal weight women. Apart from demographical and length of intervention differences they also report that physical fitness of the participants may have a role to play in the differences in observations in the studies from their laboratory. Even

though the physical fitness of the participants in Gunther et al. (2005) have been reported as higher compared to Lin et al. (2000), where sedentary women with no prior history of physical activity were enrolled on to a 2 year exercise intervention with no supplemental calcium added to their diet (mean reported habitual intake 781 ± 212 mg/d). Interestingly Lin et al. (2000) have reported calcium/Kcal ratios as significant predictors of weight change ($p < 0.01$) and decrease in fat mass ($p < 0.01$) only in women with energy intakes below the noted mean (~1893 ± 373 kcal/d) of their data set. It is therefore possible that not only the physical fitness of the participants but the exercise intervention itself lent to some of the reported changes in body composition and influenced the different outcomes of the two studies.

There seems to be two emerging trends in terms of understanding the inconsistency in reported data on the relationship between calcium intake and body compositional changes. Energy balance; high energy/high fat diets and/or exercise protocols seems to have an overriding influence on any small effect that increased dietary calcium may exert via a molecular mechanistic pathway of control of lipid metabolism and/or thermogenesis.

2.5.5 Other mechanistic concepts for the ergogenicity of calcium

2.5.5a Calcium and cellular fatigue

Muscle fatigue has been defined as a temporary loss of force or torque generating ability due to recent muscle contraction (Bigland-Ritchie and Woods, 1984). The mechanisms underlying muscle fatigue are numerous and may have their origins anywhere from central nervous system to cellular cross bridge cycling. Until recently muscle fatigue was thought to be caused by lactic acid accumulation or a lowered pH within the muscle due to a rising hydrogen ion concentration, at least in high intensity exercise (Westerblad et al., 1991). In the recent years, Cairns and Lindinger (2008) have implicated exercise induced ion shifts, water movements, physiochemical reactions and metabolic processes that lead to ion changes in compartments proximal to the sarcolemma as the major cause of fatigue (Fig. 2.5).

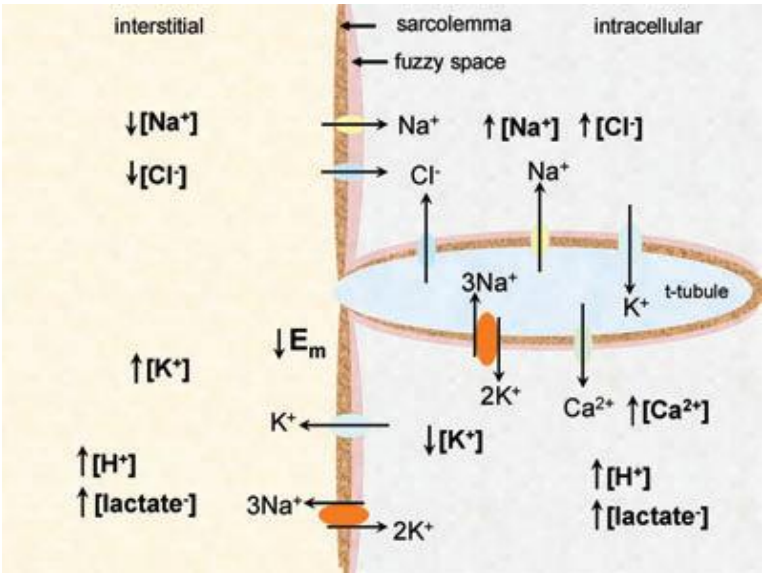


Fig. 2.13 A schematic representation of transsarcolemmal ion fluxes and ion concentration changes in various muscle compartments (interstitial, t-tubular, intracellular) during electrical stimulation or exercise (Cairns and Lindinger, 2008)

Accumulating evidence has implicated altered $_{i}[Ca^{2+}]$ regulation as a major contributor to fatigue particularly due to these changes in these ionic constituents. Specifically, increase in $_{i}[Ca^{2+}]$ levels influences slowing of relaxation in skeletal muscle which occurs due to repeated contractile activity (Westerblad and Allen, 1993). Westerblad et al. (1991) have demonstrated that calcium removal from the myoplasm was markedly reduced in fatigue. However, they have argued that this increase could be counteracted by decreased calcium sensitivity in muscle cells, but this is yet to be proved. One of the possible reasons offered for this increase in myoplasmic calcium in fatigued mammalian skeletal muscle is a reduction in the uptake of calcium by sarcoplasmic reticulum (Allen et al., 1989). Also, lowered extracellular calcium or blockers of various calcium entry pathways, exacerbates fatigue during repeated or continuous tetani (Zhao et al., 2005, Cairns et al., 1998) and raised extra cellular calcium or availability of more calcium entry pathways attenuates fatigue (Cairns et al., 1998, Zhao et al., 2005).

Numerous other studies (Allen et al., 2008b, Nummela et al., 2008, Marino et al., 2009) have demonstrated that metabolic changes during intense exercise such as accumulation of inorganic phosphates, increased ADP/ATP ratio and concurrent acidosis all play a role in reduction of maximum contraction. However, in the study by Westerblad and Allen (1991) where exercise lasted longer the muscles did not display any marked acidosis during fatigue but increased $_{i}[Ca^{2+}]$. Fatigue during such exercise is attributed to failure of calcium release from the sarcoplasmic stores (Chin and Allen, 1997, Kabbara et al., 2000) which in part can be explained by increased myoplasmic calcium levels which inhibit the feedback loop for release of calcium from sarcoplasmic stores (Allen and Westerblad 2001). However, it is the resting

$_{i}[Ca^{2+}]$ levels that normally increase alongside calcium fluxes during fatigue with repeated tetani (Allen et al., 2008a) (Fig. 2.14).

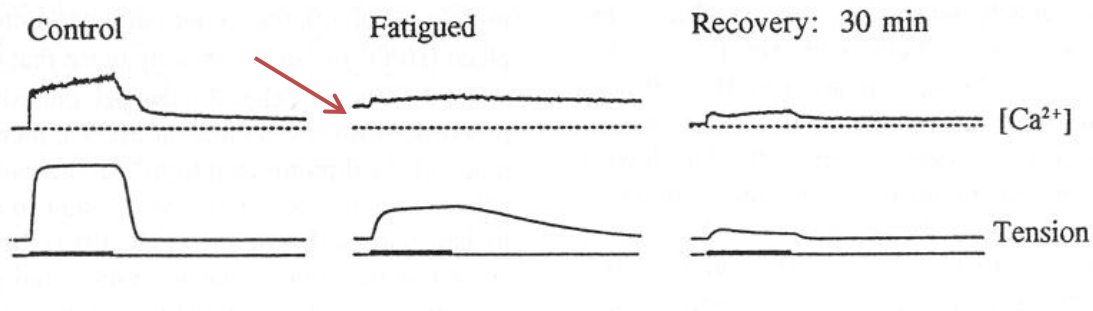


Fig. 2.14 Increased resting calcium levels in fatigued muscle fibres compared to control and return to normal levels upon recovery. Adapted from Allen et al. (2008a)

This increased resting levels of intracellular calcium upon repeated tetani is a hallmark of what is termed as low frequency fatigue (LFF) (Keeton and Binder-Macleod, 2006). This type of fatigue is characterised by slow recovery of force taking hours or days, persisting in the absence of gross metabolic or electrical disturbance of the muscle (Keeton and Binder-Macleod, 2006). It is multifactorial resulting from high intensity, moderate to high force, repetitive eccentric or stretch-shortening cycle activities (Fowles, 2006). Impairments in excitation-contraction coupling have been suggested to play a role in LFF, involving reduction in calcium release from the sarcoplasm (Edwards et al., 1975, Allen et al., 1995). Chin et al. (1997) have identified two components to this reduction in calcium release from the sarcoplasm. The first is a metabolic component which in the presence of glucose recovers within an hour, and the second is a component dependant on the elevation of the time integral of the concentration of calcium in the interstitial space of a muscle cell. This second component recovers more slowly. Westerblad et al. (1993)

have proposed a $_{i}[Ca^{2+}]$- tension relationship curve (Fig. 2.15). It proposes that since $_{i}[Ca^{2+}]$ at high frequencies is on the horizontal part of the curve, moderate falls in $_{i}[Ca^{2+}]$ have no effect on muscle tension. At low frequencies $_{i}[Ca^{2+}]$ is on the steep part of the curve, so falls in the $_{i}[Ca^{2+}]$ produce large changes in tension. The main effect of this change in $_{i}[Ca^{2+}]$ levels lead to decreased muscle forces in response to low frequency activation.

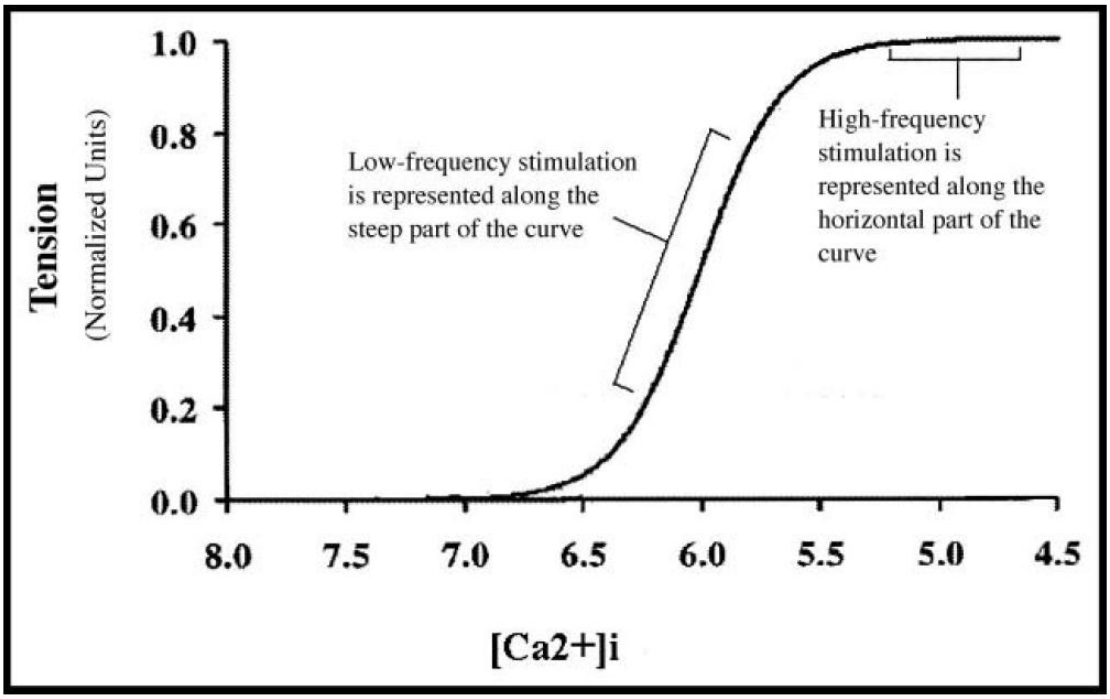


Fig. 2.15 A hypothetical $_{i}[Ca^{2+}]$- tension curve (Keeton and Binder-Macleod, 2006)

In-vivo these may be interpreted in the range of exercise more than 30 min in duration (Bigland-Ritchie and Woods, 1984). The decreased tension due to increase in $_{i}[Ca^{2+}]$ during exercise may result in the need for higher levels of activation by the CNS (Keeton and Binder-Macleod, 2006).

In summary, during submaximal exercise ion gradients show moderate perturbations (McKenna et al., 1996, Fitts and Balog, 1996, Lunde et al., 1998). However, a few

studies have demonstrated that fatigue is both task dependant and multifactorial, but ionic interactions such as increase in $_{i}[Ca^{2+}]$ levels are key factors for force loss in exercise ranging from 5 to 20 min (Allen et al., 2008b, Cairns et al., 2005). This also involves calcium–potassium interaction since elevated extracellular calcium restores force in potassium depressed muscle (Cairns et al., 1998). In addition LFF experienced by athletes due to an increased $_{i}[Ca^{2+}]$ levels during exercise may all lead to increased need for CNS drive. Therefore, if changes in $_{i}[Ca^{2+}]$ are affected via dietary calcium levels, it is possible that the fatigue mechanisms involving increased calcium levels at rest and during exercise may attenuate skeletal muscle fatigue and thus improve performance.

2.5.5b Calcium and vascular tone

McCarron et al. (1984) demonstrated that low calcium intakes in the diet were associated with a higher incidence of hypertension. Calcium intakes of less than 300 mg/d were associated with a risk of being hypertensive of 11 to 14% while calcium intakes greater than 1500 mg/d carried a risk of only 3 to 4% (McCarron et al., 1984). Zemel (2001) have also implicated a threshold intake of 600-700 mg/d calcium based on the data in the original NHANES I presented by McCarron et al. (1984) in the association of increased risk for hypertension. This association hardly seems surprising, given the role of increased $_{i}[Ca^{2+}]$ in peripheral vascular resistance (Resnick, 1992, Resnick, 1999, Kuriyama et al., 1982). Hypertensives exhibit an increase in urinary calcium losses (exacerbated by high salt diets) resulting in decreased plasma ionised calcium levels. This stimulates PTH release, which activates 1-α-hydroxylase resulting in increased 1,25-$(OH)_2$-D production (Zemel,

2001) (Fig. 2.16). This calcitropic hormone response eventually increases $_i[Ca^{2+}]$ and peripheral vascular resistance.

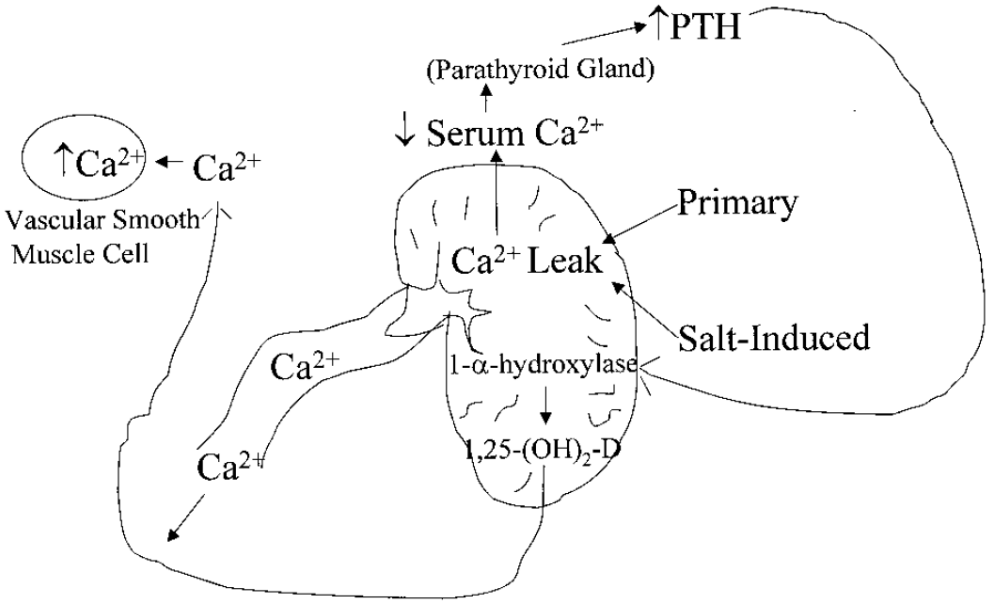


Fig. 2.16 A schematic representation of the role of calcium in vascular resistance (Zemel, 2001)

Increasing dietary calcium prevents this sequence in a similar mechanistic pathway described earlier. Therefore the role of increasing dietary calcium can be important in maintaining vascular tone and decreased peripheral resistance during exercise. This will have implications on maintaining blood flow and oxygen supply further improving performance.

2.6 Calcium and exercise performance

Most data on calcium homeostasis in the blood during exercise is derived from studies looking at bone health and exercise. There are not many specific studies looking at calcium intakes and its relation to substrate metabolism during exercise. White et al. (2006) have reported no significant treatment effect of acute calcium (500 mg) intervention on endurance trained female runners. On a 90 min glycogen depletion trial followed by a 10 km time trial performance the researchers found no difference in fat oxidation rates of appearance of glycerol in the blood stream nor changes in respiratory exchange ratios during the performance test. Acute calcium intake also did not seem to have any influence on time to finish on the 10 km time trial. Melanson et al. (2005) used a 70% $\dot{V}O_{2peak}$ protocol in their seven day high and low dairy intervention study to achieve a ~400 Kcal energy deficit. They found a significant increase in 24 hour fat oxidation rates when their participants were on the exercise protocol between high dairy (HD~1400 mg/d) and low dairy (LD~500 mg/d) groups (HD = 136 ± 13 g/d, LD = 106 ± 13 g/d; $p < 0.05$). They have not looked at any performance measures and perhaps it would be speculative to infer that the increase in fat oxidation rates would translate into glycogen sparing action and thus would have improved performance in this experiment, especially since this population were untrained individuals. The main role of lipid mobilisation during exercise is played by β-adrenoreceptor-mediated activation of hormone sensitive lipase in the adipocytes (Holloszy and Coyle, 1984). And since Xue et al. (2001) have demonstrated that $_i[Ca^{2+}]$ activates PDE 3B reducing the intracellular pool of cAMP and thereby suppressing the phosphorylation of HSL, there seems to be merit in a high calcium diet and its purported effects on increased availability of NEFA, which in turn should increase fat oxidation during endurance exercise.

There are no studies that have looked at ionised calcium in blood in relation to exercise with a calcium supplementation intervention, either acute or chronic. Men'shikov (2004) have reported an increased availability of NEFA with a corresponding decrease in plasma ionised calcium after exercise in trained athletes as compared to untrained controls when doing a endurance cycle ergometer test of 90 min duration without any calcium related intervention however. In addition, they have reported a multiple correlation between post-exercise plasma NEFA, ionised calcium and oxygen consumption in athletes ($r = 0.72$; $p < 0.05$) suggesting an involvement of calcium in NEFA metabolism. Thus even though there does not seem to be a direct effect of acute calcium load on performance parameters, the influence of calcium on body composition may still be an avenue for further research with respect to its ergogenic properties.

2.7 Summary and research questions

Calcium plays a variety of roles in the human metabolism (Fig.2.17). *In-vitro* and animal studies have suggested a strong correlation between increased $_{i}[Ca^{2+}]$ and fat metabolism with a concurrent chronic effect on body weight and composition.

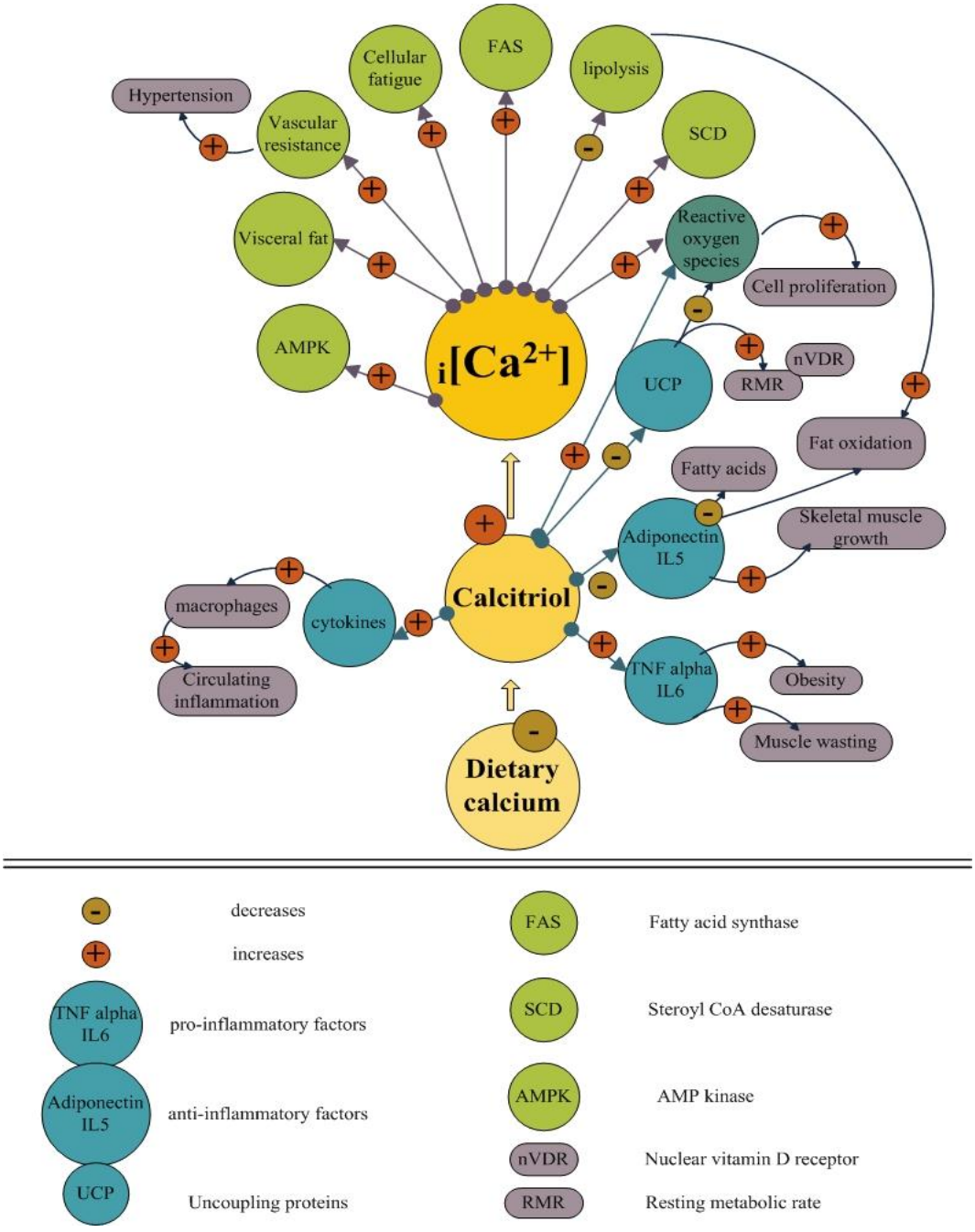


Fig. 2.17 Schematic representation of the role of dietary calcium in metabolism

However, studies conducted in humans are inconclusive towards supporting those results. With calcium levels being tightly regulated in the body, any intervention designed to study its specific role in energy metabolism has obvious challenges in terms of methodology and control of confounding factors. Over and above differences in the source of calcium (dairy and supplemental forms), the measurement of energy expenditure in free living organisms (especially in simulated laboratory conditions) all further complicate the comparison of different experimental conditions. As seen from the evidence presented that even though experiments in total energy expenditure are inconclusive in supporting the hypothesis of calcium aiding EE, there seems to be an emerging bank of data favouring the shift in substrates towards increased fat oxidation with higher dietary calcium intakes especially under energy deficit conditions.

Studying the calcium-induced shift of substrates during exercise with relation to training adaptations in athletes would enhance our understanding in this research field immensely. Trained athletes are better able to utilise fat as a source of energy during sub-maximal exercise (Holloszy and Coyle, 1984). If dietary calcium plays a role in lipolysis and thus increasing the availability of NEFA, it may aid in increased fat oxidation rates during a given exercise bout. This in turn may help spare glycogen in certain endurance events, leading to performance enhancement and/or delay in fatigue. These metabolic shifts may help change the athlete's body composition favourably towards higher fat loss.

Thus in the view of the literature presented, the following experimental chapters will address the aims and objectives laid down in Chapter 1.

CHAPTER 3

GENERAL METHODS

This chapter outlines the common methodologies undertaken for all studies. Where methods for the various studies differ specific methodologies are then described in the relevant chapters.

3.1 Participant recruitment

All investigations were approved by the Ethics Health and Safety Committee, Faculty of Science and Technology, University of Central Lancashire. Prior to participation in all the studies, each participant was provided with an information sheet (appendix C and D) outlining the purpose of the research, how it was to be undertaken, the potential risks and benefits, the points and sources of registering complaints and grievances regarding any aspect of the research and anticipated degree of commitment.

Inclusion criteria involved participants being healthy and free of known cardiovascular, respiratory and circulatory dysfunction, not taking calcium supplements nor vitamin D preparations, anticonvulsant medications, diuretics, adrenocorticosteroids, oestrogens or other drugs that could alter calcium metabolism

for the past 12 months. This was assessed via a health screening questionnaire (appendix E) and Physical Activity Readiness – Questionnaire (PAR-Q) (appendix F). Minimum training load and volume specifications of the recruitment criteria are defined individually in all studies as they varied in these different trials. Prior to testing all participants returned a signed copy of their informed consent (appendix G and |H) to participate in the trials.

3.2 Equipment

3.2.1 Cycle ergometry

A stationary electromagnetically braked cycle ergometer (SRM ergometer; Schoberer Rad Messtechnik, Jülich, Germany) was used in studies 2 and 3. This system is a crank-based device that measures the mechanical Power Output (PO) using 20 strain gauges attached to components inside the crank. The measured torque and cadence values were digitalised inside the crank and converted to a high frequency, pulse-width modulated electrical signal. The data was transmitted to a microcomputer on the handlebar and also displayed on a computer using the SRMWin software (Schoberer Rad Messtechnik, Jülich, Germany), where the torque was averaged over each complete pedal revolution and multiplied by the cadence to calculate the PO reading using the following equation -

PO = ([measured frequency – zero offset frequency] x cadence x 2π) / (slope x 60)

The slope for each SRM crank dynamometer is calculated dynamically at the SRM factory. Positions of the handlebars, seat height and crank length were adjusted to the measures used by the athletes own racing bikes. The SRM crank system has been

previously confirmed as a valid and reliable device for determination of PO in cycling (Balmer et al., 2000). During each exercise test, torque and angular velocity data from the bicycle crank were relayed to a power-control unit attached to the ergometer (Fig. 3.1). Subsequent power output data were obtained at 1 sec intervals from the power-control and transferred to the SRM software and downloaded to a personal computer (Appendix L).

Fig. 3.1 Powercontrol unit on the SRM ergometer

Reliability of the power measurements was calculated (n = 2) by performing two graded incremental trials (20 W/min until voluntary exhaustion) on the SRM ergometer four days. Data points at 60s average at each min were used to calculate the change in mean, typical error (TE) and Pearson's product moment correlation coefficient (R^2) (table 3.1). Bland and Altman plots were produced as described in Lamb (1998) (Fig. 3.2). The data in the Bland and Altman plots is presented as log transformation due to its heteroskedastic nature.

Table 3.1 Change in mean, typical error and correlation coefficient for power output Units ± SD (95% CI upper to lower limits)

	Change in mean	Typical error	(R^2)
PO	-7.8 ± 4.6 (-10.5 to -5.1)	3.3 (2.3 to 5.7)	0.99 (0.98 to 1.00)

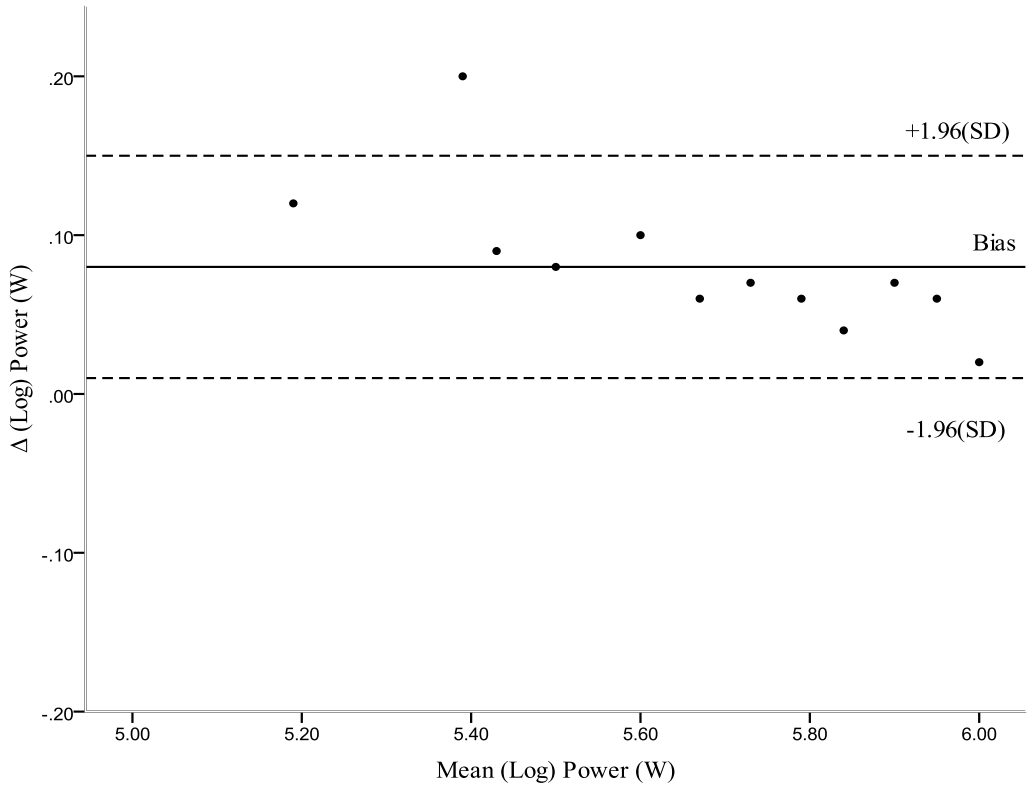


Fig. 3.2 Bland and Altman plots of mean (log) power output (W) of graded incremental tests (n = 2)

3.2.2 Respiratory gas collection and analysis

Expired gas samples were collected throughout using the Cortex Metalyzer 3B laboratory Breath-by-Breath spiroergometry System (Cortex Biophysik GMBH, Version ML3B 2.1). During cycling, the expired air was collected via a bi-directional digital volume transducer attached to a facemask of appropriate size for the participant which was secured over the mouth and nose ensuring all gas breathed in and out passed through the flow sensor and sample line. The expired air was continuously and simultaneously measured for volume; O_2 and CO_2 concentrations in conditions of standard temperature pressure and dry (STPD). The CO_2 output was determined by an infra-red sensor and O_2 uptake during each breath was determined by an electrochemical cell (Cortex Biophysik GMBH). Data sampled were transferred breath by breath to a personal computer (PC) for immediate display. The data recorded was saved in the internal databases of Metasoft V3.7. The Metalyzer underwent a 2 point calibration immediately before each test with the use of ambient air (O_2 20.93% and CO_2, 0.03%) and a gas of known composition (O_2 15%, CO_2 5% BOC, Guildford, UK). The DVT turbine, digital volume transducer was calibrated prior to each test with a three litre calibration syringe (Hans Rudolf). Heart rate was recorded throughout using short range telemetry. The chest transmitter belt (Polar Electro T60, Kempele, Finland) was placed inferiorly to the xiphisternal joint. Data was transmitted to a heart rate (HR) receiver housed in the portable body of the Metalyzer 3B. Data was recorded breath by breath and average calculated for every 30 sec for the purpose of analysis.

Reliability of the gas analysis measurements was calculated (n = 2) by performing two graded incremental trials (20 W/min until voluntary exhaustion) on a cycle ergometer four days apart. The participants met all conditions described in section

3.1 prior to the test. Data points at 60 sec average at each min were used to calculate the change in mean, typical error (TE) and Pearson's product moment correlation coefficient (R^2) (table 3.2). Bland and Altman plots were produced for $\dot{V}O_2$ (Fig. 3.3) and $\dot{V}CO_2$ (Fig. 3.4) after the data was participant to all fulfilment of all conditions as described in Lamb (1998). The data in the Bland and Altman plots is presented as log transformation due to its heteroskedastic nature.

Table 3.2 Change in mean, typical error and correlation coefficient for gas exchange analyser. Units ± SD (95% CI upper to lower limits)

	Change in mean	Typical error	(R^2)
$\dot{V}O_2$	-0.70 ± 3.7 (1.05 to -2.45)	2.68 (3.92 to 2.04)	0.93 (0.97 to 0.82)
$\dot{V}CO_2$	-0.10 ± 0.5 (0.14 to -0.33)	0.36 (0.52 to 0.27)	0.89 (0.96 to 0.76)

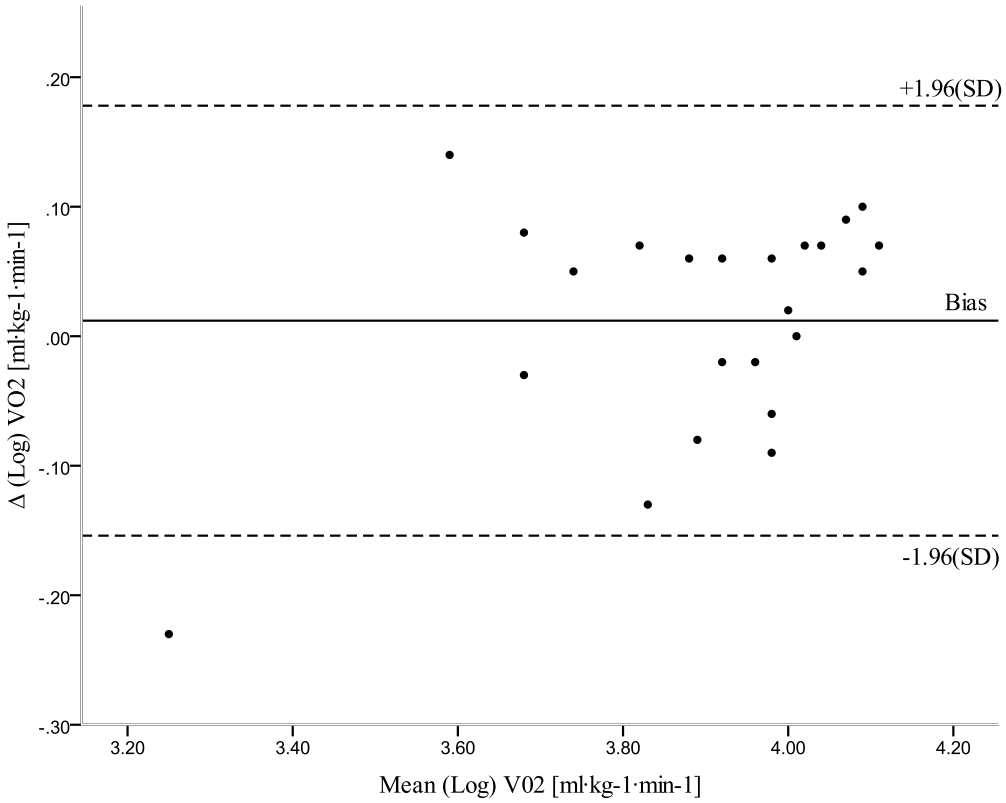


Fig. 3.3 Bland and Altman plots of mean (log) oxygen consumption (ml/kg/min) of graded incremental tests (n = 2)

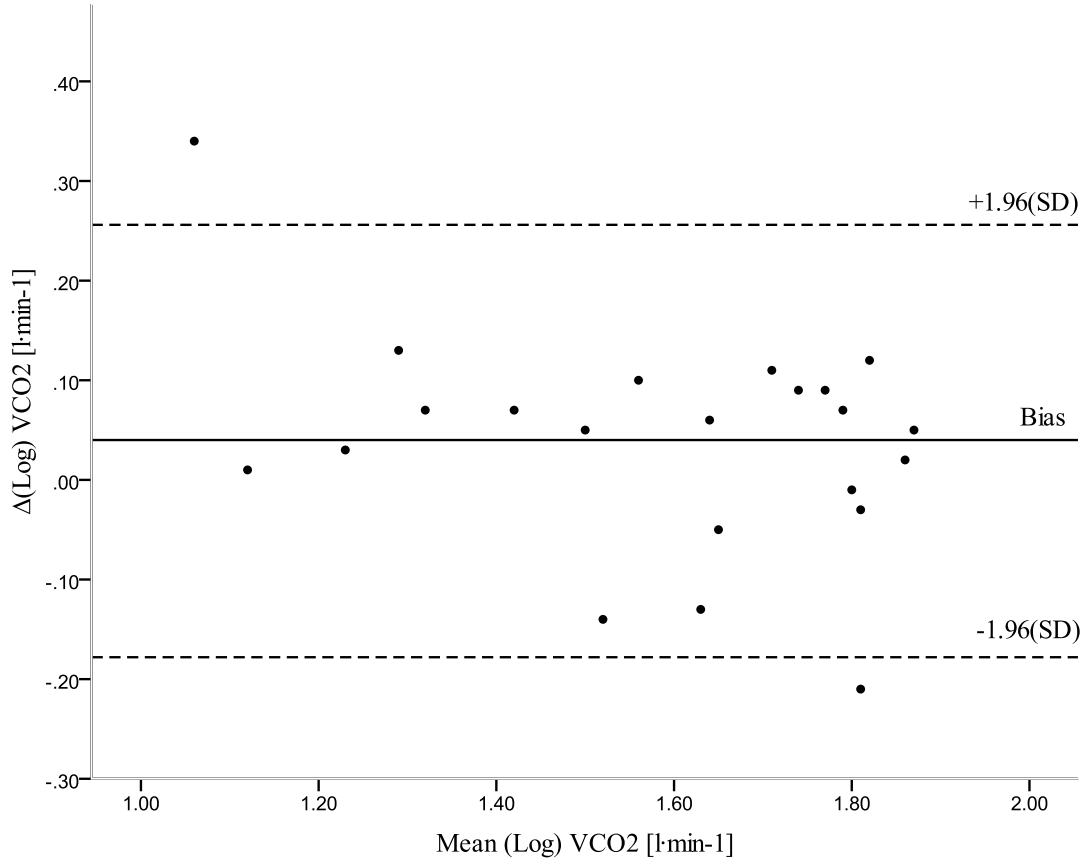


Fig. 3.4 Bland and Altman plots of mean (log) carbon dioxide production (l/min) of graded incremental tests (n = 2)

To determine the variability in the gas analysis measurements during the average testing time period for the trials (~80 min) calibration gases (O_2 15%, CO_2 5% BOC, Guildford, UK) were measured immediately after calibration and repeated after the machine had been left running for 80 min. The readings for oxygen and carbon dioxide were same at 0, 40 and 80 min.

3.2.3 Rating of perceived exertion

Rating of perceived exertion (RPE) was measured using the 15 point Borg Scale (Borg, 1973). The scale runs from 6 to 20 with 6 being very, very light and 20 being very, very hard. Participants were instructed to non-verbally score their perception of exertion immediately before every blood collection stage in all studies.

3.2.4 Haematological variables

3.2.4a venous sampling

A 3 ml venous blood sample was taken from the median cubital vein in the antecubital fossa using a syringe containing ethylenediaminetetraacetic acid (EDTA) as an anti coagulant (S-Monovette, Sarstedt, Germany) for analysis at rest before the start of each trial. All samples were transferred to Eppendorf tubes, mixed thoroughly and immediately centrifuged at 10,000 rpm for 6 min and the supernatant was frozen at -80°C until further analysis for plasma intact parathyroid hormone (iPTH) and NEFA (study 1)

i. Plasma intact parathyroid hormone

iPTH concentration in the plasma was determined using a two-site quantitative immunoenzymometric assay kit (OCTEIA® Intact PTH, Immunodiagnostic Systems Ltd., Tyne and Wear, UK). The procedure consisted of incubation of duplicates of 200 µl of the sample, six standards (lyophilised porcine plasma containing PTH with 0.09% (w/v) sodium azide in varying concentrations of 0.0 pmol/l, 1.5 pmol/l,

4.8 pmol/l, 7.5 pmol/l, 17.3 pmol/l, 39.1 pmol/l) and controls (distilled water) in antibody coated (N-terminal specific mouse monoclonal anti-PTH) polystyrene micro titre wells for four hours at 4°C before aspirating and manually washing three times by adding 300 µl of wash solution (phosphate buffered saline) and decanting to remove unbound sample. The micro titre plate was then inverted firmly on an absorbent tissue to remove excess wash solution. The wells were then incubated at 4°C overnight with 200 µl of enzyme conjugate (affinity purified goat anti-PTH coupled to horseradish peroxidase). Following a further aspiration and wash step, colour was developed using 200 µl liquid TMB substrate reagent and the wells were incubated at room temperature for 30 min. 100 µl of stop solution (0.5M hydrochloric acid) was used to stop the reactions. The micro titre plate was then read immediately in a plate reader (SpectraMax Plus384 Microplate Spectrophotometer, CA, USA) at 450 nm. The colour intensity developed is directly proportional to the amount of PTH in sample.

Calculation of iPTH concentration (pmol/l) from mean absorbance of the duplicates was read on a calibration curve prepared by plotting the mean absorbance of each standard concentration on the x axis against concentration of iPTH on the y axis (Fig. 3.5). The %CVs of the standards are presented in table 3.3.

Quality control of the assay was performed by measuring the within-run CVs which ranged from 0.62 – 4.13% (Appendix B) and the between-run CV was 6.81%

Table 3.3 Standard absorbance and %CV for iPTH assay

Std Concentration (pmol/l)	absorbance	absorbance	mean absorbance	StdDev	%CV
0	0.066	0.065	0.066	0.001	1.1
1.5	0.107	0.109	0.108	0.001	1.3
4.8	0.198	0.188	0.193	0.007	3.7
7.5	0.309	0.32	0.315	0.008	2.5
17.3	0.605	0.617	0.611	0.008	1.4
39.1	1.347	1.359	1.353	0.008	0.6

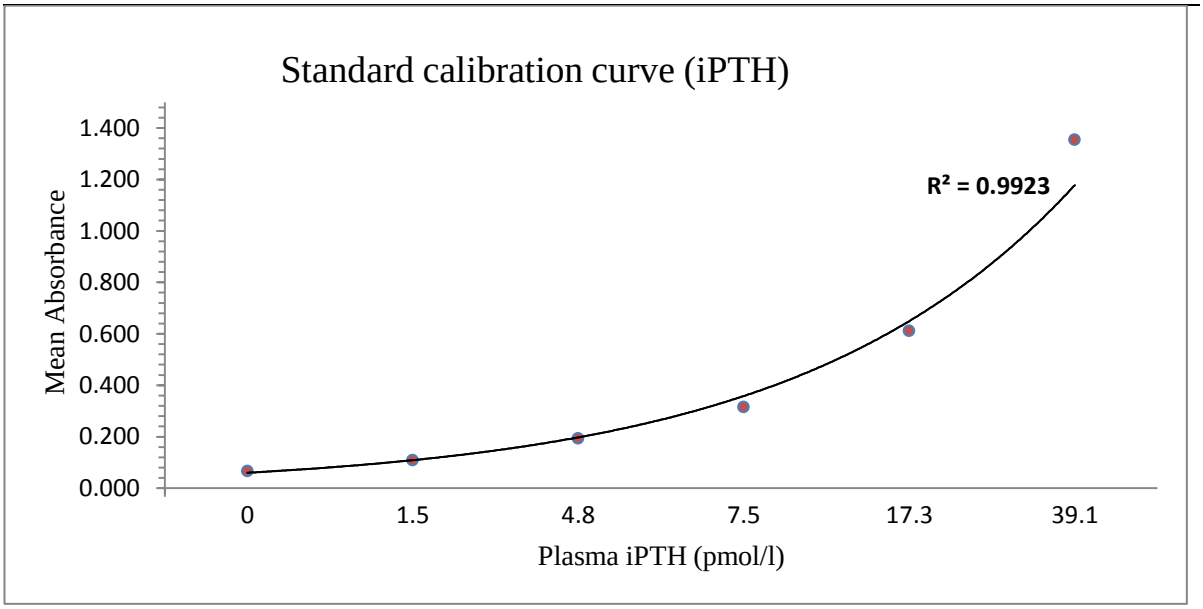


Fig. 3.5 Standard calibration curve for iPTH assay

ii. Plasma non esterified fatty acids

Plasma NEFA concentration was determined using a NEFA C test kit (WAKO Chemicals GmbH, Neuss, Germany). The half-micro test determined NEFA concentrations by an optimised enzymatic colorimetric assay. The procedure involved mixing 5 µl of plasma (sample) or 5 µl of distilled water (control) with 100

µl of surfactant buffer solution containing ATP, coenzyme A, ascorbate acetate, acyl-CoA-synthetase (Acyl CS) and. Once the mixture had been incubated in a water bath at 37°C for 10 min, 200 µl of acyl-CoA-oxidase and peroxidase in surfactant (3-methyl-N-ethyl-N-(β-hydroxyethyl)aniline) solution was added and incubated for a further 10 min at 37°C. The absorbance of the sample (E_1) and control (Ca-1) were measured immediately after using a spectrophotometer (SpectraMax Plus^{384} Microplate Spectrophotometer, Molecular Devices, CA, USA) set at a wavelength of 546 nm. Measurements were conducted in triplicates and a mean of the readings was taken for calculation. Since our samples were not strongly lipemic, icteric or haemolytic a sample blank value correction was not applied. The concentration of the NEFA (mmol/l) was determined using the following calculation:

$$\text{NEFA (mg/dl)} = E_s \times \frac{\text{Conc}_{std}\ \text{(mg/dl)}}{E_{std}}$$

Where [NEFA] is the concentration of free NEFAs in mg/dl which was then converted to mmol/l (conversion factors: mg/dl x 0.035 = mmol/l)

E_s is [E_1-Ca-1]

Conc_{std} was Oleic acid (28.2 mg/dl i.e. 1.0 mmol/l)

E_{std} is the absorbance of the standard at 546 nm

Quality control of the assay was performed by measuring the within-run CVs which ranged from 0.90 – 5.79% (Appendix B) and the between-run CV was 4.69%

3.2.4b Capillary sampling

Blood samples were taken from either the index or the middle finger of the preferred hand. The finger was first cleaned using a Steret alcohol wipe (Southern Syringe Ltd., Manchester, UK) to ensure the area was clean of any debris. The finger was then punctured using a BD Microtainer® Genie™ blade lancet (2.00 mm x 1.50 mm, Blue) (Becton, Dickinson & Co., Plymouth, UK) and the first drop of blood wiped off to ensure any remaining alcohol did not affect the sample. A 5 µl sample was taken for measurement of whole blood lactate (study 1 and 2) and a further 5 µl sample was taken for measurement of blood glucose (study 1 and 2). A 300 µl sample was collected in a Microvette® CB300 tube (Sarstedt Ltd., Leicester, UK). It was mixed thoroughly and immediately centrifuged (Micro Centaur, MSE Ltd. London, UK) at 10,000 rpm for 6 min and the supernatant was frozen at -80°C until further analysis for total plasma calcium, triacylglycerides, glucose and NEFA.

i. Whole blood lactate

Whole blood lactate was measured using a lactate test meter (Lactate Pro, Akray, Kyoto, Japan). This is a hand-held portable analyser capable of measuring whole-blood lactate in the field. Capillary blood was drawn as explained above and then applied to a reagent strip (lactate Pro Test Strip, Akray, Kyoto, Japan). Lactate in the sample reacts with potassium ferricyanide and lactate oxidase to form potassium ferrocyanide and pyruvate. Upon the application of a given voltage, ferrocyanide is oxidised, releasing electrons and creating a current. This current is measured amperometrically and is directly proportional to the lactate concentration of the blood sample. The Lactate Pro is supplied with a Check Strip (to confirm that the

analyser is operating correctly) and a Calibration Strip that provides a non-quantitative indication of instrument accuracy. This method has been previously validated (Mc Naughton et al., 2002, Pyne et al., 2000, Makita and Satomi, 1997). They have all found strong correlations between ($r > 0.96$) (Pyne et al., 2000), ($r = 0.98$) (Mc Naughton et al., 2002) and ($r = 0.99$) (Makita and Satomi, 1997) between the Lactate Pro and other existing analysers. The limits of agreement of less than 2.0 mM through the physiological range of 1.0 – 18.0 mM (Pyne et al., 2000) underlie the suitability of this method for our current experimental conditions as all tests conducted are in the submaximal range of exercise intensity, where blood lactate are unlikely to rise over 18.0 mM (Telford et al., 1988)

ii. Total plasma calcium

Total plasma calcium was measured using Dr. Lange Miniphotometer plus LP 20 (Hach Lange GmbH, Berlin, Germany) with Dr. Lange calcium cuvette test kit (LKM 145). In alkaline buffer solution calcium forms a purple dye with o-Cresolphthalein this is measured photometrically. Contents of cuvette (o-Cresolphthalein complexion 0.16 mmol/l contained in LKM 145) were dissolved in 2.0 ml buffer solution (glycine buffer, pH 10.3: 300 mmol/l). Then 50 µl sample and blank (50 µl distilled water) was pipetted into the dissolved reagent, which was then mixed gently and analysed against blank within 30 min at 578 nm at room temperature (20 to 25°C). Total plasma calcium concentration was recorded in mmol/l. Quality control of the assay was performed by measuring the within-run CVs which ranged from 0.0 – 0.54% (Appendix B) and the between-run CV was 2.36%

iii. Plasma triacylglycerides

Plasma triacylglycerides was measured using Dr. Lange Miniphotometer plus LP 20 (Hach Lange GmbH, Berlin, Germany) with Dr. Lange triglyceride cuvette test kit (LKM 227). This is an enzymatic colorimetric test. Plasma triglycerides are hydrolyzed to glycerol and free NEFAs by lipase. In the presence of ATP and glycerol kinase (GK), the glycerol is converted to glycerol-3-phosphate. The glycerol-3-phosphate is then oxidized by glycerol phosphate oxidase (GPO) to yield Dihydroxyacetone phosphate and hydrogen peroxide. The condensation of hydrogen peroxide with 2, 4 dichlorophenol and 4-aminophenazone in the presence of peroxidase (POD) produces a red coloured quinonimine dye which is read at 520 nm. The intensity of the coloured complex formed is directly proportional to the triacylglyceride concentration of the sample.

10 µl sample and blank (10 µl distilled water) was pipetted into the LKM 227 cuvette containing buffer solution (lipoprotein lipase, peroxidase and 2, 4-Dicholorphenol) and the absorbance measured (A0) at 520 nm. The protective foil was then removed, the cap containing starting reagents (ATP, GPO, peroxidase and glycerokinase) was inverted and the reactions catalysed my mixing the contents thoroughly. The cuvette was then immediately inserted into the Miniphotometer and absorbance read (Pl-1) at 520 nm. The inbuilt software made the necessary adjustments from A0 and Pl-1 and displayed plasma triacylglycerides in mmol/l. Quality control of the assay was performed by measuring the within-run CVs which ranged from 0.08 – 0.25% (Appendix B) and the between-run CV was 1.30%

iv. Plasma glucose

Plasma glucose was measured using Dr. Lange Miniphotometer plus LP 20 (Hach Lange GmbH, Berlin, Germany) with Dr. Lange glucose cuvette test kit (LKM 141). Glucose is determined after enzymatic oxidation in the presence of glucose oxidase (GOD). The formed H_2O_2 reacts under catalysis of peroxidase (POD) with 2, 4-Dicholorphenol and 4-aminophenazone to form red quinonimine dye. The intensity of the colour is proportional to the glucose concentration in the sample.

10 µl sample and blank (10 µl distilled water) were pipetted into the LKM 141 cuvette containing colour reagent (Phosphate buffer, pH 7.8, mutarotase, 2, 4-Dicholorphenol and 4-aminophenazone) and the absorbance measured (A0) at 520 nm. The protective foil was then removed, the cap containing starting reagents (glucose oxidase and peroxidase) was inverted and the reactions catalysed my mixing the contents thoroughly. The cuvette was then immediately inserted into the Miniphotometer and absorbance read (Pl-1) at the end of two minutes at 520 nm. The inbuilt software made the necessary adjustments from A0 and Pl-1 and displayed plasma glucose in mg/dl. Quality control of the assay was performed by measuring the within-run CVs which ranged from 0.13 – 0.87% (Appendix B) and the between-run CV was 1.39%

3.2.5 Anthropometry

All anthropometric measurements were made in a resting state according to established procedures (Eston and Reilly, 2001).

3.2.5a Stature and Total body mass

Stature was measured using a stadiometer to the nearest 0.05 m (Harpenden, Avery Ltd, Birmingham, UK). Total Body Mass was determined using a balance beam scale to the nearest 0.1 kg (Avery Type 3306, Avery Ltd, Birmingham, UK).

3.2.5b Body composition

i. Air-displacement Plethysmography

Body composition and total mass (studies 2 and 3) were determined using air-displacement plethysmography (Bod Pod Express, Life Measurement Incorporated, Concord, USA). The test is conducted after of a 2 point calibration. The weighing scale was calibrated with reference weights of 2x10 kg and the air volume in the empty front chamber measured against reference gas cylinder of 50 kg (Life Measurement Incorporated, Concord, USA). After the calibration the participant's mass is recorded using the same scale and volume, which is determined by sitting inside the Bod Pod chamber. The Bod Pod consists of two chambers. The front, or Test Chamber, is where the participant is asked to sit in a swimsuit and swim cap only. This is done in order to keep air trapped between clothing and hair to a minimum as that would interfere with the readings. In addition any jewellery,

spectacles, and any other clothing or accessories are removed prior to testing. The seat in the front chamber forms a common wall separating it from the rear, or Reference Chamber. During data collection of the volume measurement, the chamber door is secured by a series of electromagnets and a rubber gasket. A Diaphragm is mounted on the common wall, which oscillates during testing. This causes small changes in volume inside the chamber, of which the pressure response to these small volume changes is measured (Fig. 3.6). This is done by measuring the interior volume of the empty Bod Pod chamber, then measuring it again when the participant is seated inside. The participant's total body volume is obtained by subtraction (Life Measurement Incorporated, Concord, CA, USA). Once mass and volume are determined body density is calculated and the relative proportions of fat and fat-free mass are determined using the Siri equation (Siri, 1956). This method of body composition has previously been validated elsewhere (Collins et al., 1999; Fields et al., 2000). All measurements were taken in triplicates and the mean of the three readings used for all calculations.

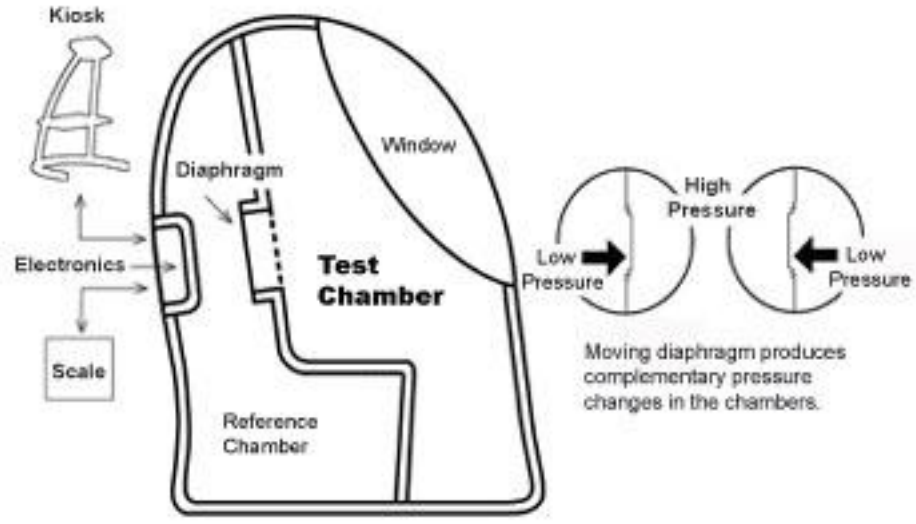


Fig. 3.6 Schematic representation of the working of the BodPod

3.2.6 Nutritional and Physical Activity Analysis

3.2.6a Seven day diet diaries

All participants were asked to provide a seven day diet diary for all studies. They completed the record during one typical week and one typical weekend prior to beginning testing. A representative page from the diary is presented in appendix H. Even though the seven day diet dairy has been validated as a sensitive and fairly accurate measure of dietary analysis (De Castro, 1994), in order to keep the motivation of the participants' reporting accuracy as high as possible a commitment to providing a detailed nutrient composition analysis of their reported diets was offered as a reward for adherence. The participants were informed that the accuracy of their reports is exactly linked to the accuracy of their records. To aid them in providing correct estimates of portion sizes, sample size pictures were provided along with the diary (appendix I). They used these when an accurate weight of the food consumed was not available. They were also instructed to report any uneaten food that was left on the plate. They were informed that record keeping often produces a change in nutrient intake (Balogh et al., 1971; Hegsted, 1975) and they should eat normally as their dietary composition report will only be of use to them if it accurately reflects their normal intake.

Nutrient analysis was conducted using the WinDiets Research software (Robert Gordon University, Scotland, UK). The Nutrients included in this analysis for the purpose of participant reports were dietary energy, protein, total fat, saturated fat, monounsaturated fat, polyunsaturated fat, dietary cholesterol, carbohydrate, total sugars, alcohol, fibre (non-starch polysaccharides), calcium, retinol, carotene, thiamin, riboflavin, niacin, vitamin B6, folate, vitamin Ca-12, vitamin C, vitamin D

and vitamin E. However, only energy, total fat, protein, carbohydrate and calcium values were used in the analysis for the purpose of the project.

During the laboratory tests no food intake was allowed, whereas fluid intake such as tap water was permitted *ad-libitum*. Sports drinks with carbohydrates and/or electrolytes were not permitted.

3.2.6b Quantification of training

The most valid measure for quantification of training has been recognised as a measure of the sum of duration multiplied by the intensity, which is proportional to energy expenditure (Hopkins et al., 1999). In order to achieve this all participants were asked to produce a training log. Heart rate was recorded for seven days (a typical week and a typical weekend) during the training sessions in study 1. Ideally it was to be the same week that was used to record food intake. In study 2 and 3, participants were encouraged to keep a training log for the entire four weeks of supplementation, although a minimum of seven days was considered essential. Training impulse Score (TRaining IMPulse Score) method (Banister et al., 1975) was used to quantify the training of all the participants during the trials. The dose response relationship explored in Bannister et al. (1975) indicates a direct relationship between training stimulus and adaptations in humans.

The training load can be expressed as –

Training load = Intensity x Duration

This helps quantify the training stimulus as a composite of external loading and physiological response, multiplying the training load (stress) by the training intensity

(strain) (Jobson et al., 2009a). Training intensity was identified using HR. Five HR zones based on HR_{peak} have been identified i.e. 50 - 60%, 60 -70%, 70 – 80%, 80 - 90%, 90 – 100% of HR_{peak} (Edwards, 1994, Jobson et al., 2009b, Foster et al., 2001). Each of these zones were given a multiplier score (50 - 60% - 1, 60 -70% - 2, 70 – 80% - 3, 80 -90% - 4, 90 – 100% - 5 (Foster et al., 2001). An exercise for each session was then computed by multiplying the duration of the session by the relevant HR zone multiplier. Mean weekly load, monotony (daily TRIMP x SD) and strain (monotony x load) were then compared between the two sets of trials.

3.2.7 Supplementation formulation and conditions

3.2.7a Calcium citrate capsules

100% Calcium citrate powder was used for all interventions (NOW Foods, Illinois, USA). Calcium complexed with citric acid is a white amorphous, tasteless and odourless powder and this product contained no sugar, salt, starch, yeast, wheat, gluten, corn, soy, milk, egg, shellfish or preservatives. Calcium citrate contains approximately 21-24% elemental calcium (Levenson and Bockman, 1994). 3 g of the calcium citrate contained ~700 mg of elemental calcium (23.3% elemental calcium). Based on this an individual dose of 4.3 g/d of calcium citrate for four weeks was prepared. This amounted to four capsules per participant per day (1090 x 4 = 4360 mg). Capsules were made with vegetarian shells (uk-capsules.com) made from hydroxypropylmethyl cellulose (pine wood fibres). Size “00” capsules were filled (filled weight range 540 – 1090 mg) with a capsule filler (Cap·M·Quik, California, USA.) and Tamper (Cap·M·Quik, California, USA.).

The process was conducted by a single technician (author), to minimise the variations in amount of powder in each capsule. Capsules were made in room temperature (19 - 22°C) on a bench first cleaned with sterile solution and then wiped dry with tissues. Nitrile gloves were used throughout the process of handling all materials and equipment. The steps involved were as follows –

Assembly: The base of the capsule holder was placed on the bench lined with tissues. Spacers (Fig. 3.7a) were placed on end posts facing centre. And the holder was set on the four corner posts resting on spacers above base.

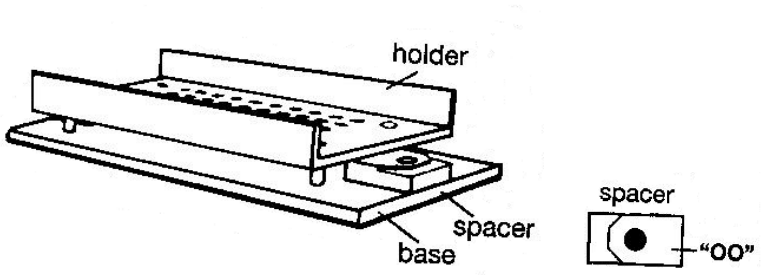


Fig. 3.7a Assembly of the capsule making equipment

Loading: Capsule shells were manually separated and the longer halves were placed in the holes, making sure the top of the shells were below the surface of the holder (Fig. 3.7b).

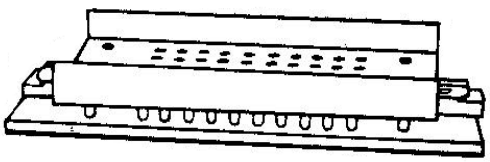


Fig. 3.7b Loading of the capsule making equipment

Filling: The calcium citrate powder was loaded on to one side of the holder and spread evenly with a flat card provided with the equipment, filling all capsules to the maximum limit (Fig.3.7c).

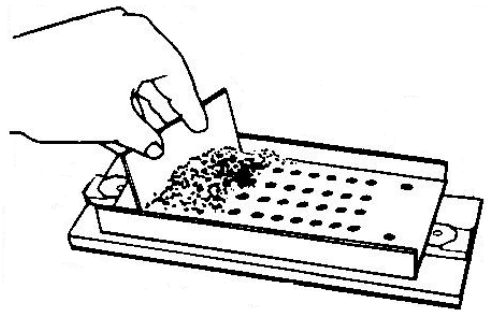


Fig. 3.7c Filling of the capsule making equipment

Tamping: A tamper was then used to simultaneously pack the contents of the capsules and remove excess powder from the surrounding area on the holder (Fig. 3.7d). Each session was tampered and refilled twice to maintain similar packed volume and achieve maximum filled weight capacity.

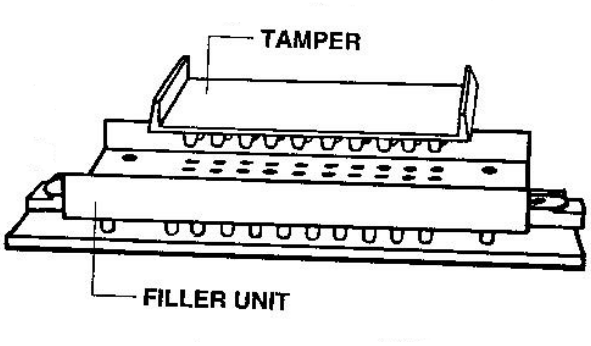


Fig. 3.7d Tamping of the capsule making equipment

Capping: The spacers were then turned 90°. This makes the holders fall down exposing the upper half of filled capsules. Top caps were then fixed manually to each capsule (Fig. 3.7e) and the ready capsules tapped and removed from holder and stored in airtight containers.

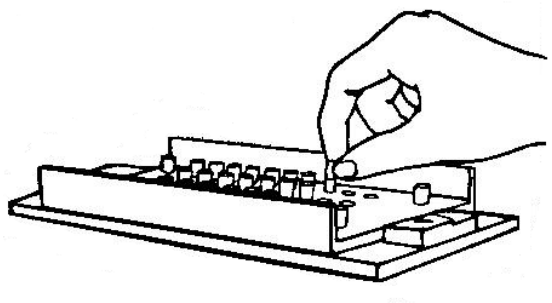


Fig. 3.7e Capping of the capsules

Once capsules were made, a random selection of 10 capsules from each batch was weighed individually on a beam balance (Adam PGL precision balance) and weight recorded for variance. Similarly, the variance for between batches was calculated and adjusted for. A total of 26 batches per intervention were made.

3.2.7b Placebo capsules

Micro crystalline cellulose (Avicel PHlOP, Honeywell and Stein, Surry, UK) was used as placebo. Microcrystalline cellulose is indigestible and widely used in research as placebo (Kalliomäki et al., 2001, Walter et al., 2000). The physical properties of the cellulose made it ideal as the two sets of capsules (calcium citrate and placebo) were indistinguishable from each other. This was 'sight' tested on 10 individuals prior to being used in all the interventions. The method of preparation of

the placebo capsules was the same as described in 3.2.7a for the calcium citrate capsules. the weight of the placebo capsules were participanted to the same variance testing as the calcium citrate capsules for within and between batch weights of individual capsules. Further, the variance between the placebo and the calcium citrate tablets was calculated for weight parity among all capsules.

3.2.7c Conditions for ingestion

All participants were asked to ingest 2 capsules (1090 mg calcium citrate [one capsule content] contains 259 mg of elemental calcium) x 2 times /d with water or fruit juice in a post prandial state of at least 20 min as there is conflicting data on influence of meal conditions on the absorption of calcium in the gut (Heaney et al., 1990b, Smith et al., 1987). The load of calcium dictates absorption rates in most healthy individuals and a 400-500 mg load is considered as optimal for maximum absorption (Heaney et al., 1990a) and thus a single dose of ~518 mg was given twice per day. The participants were also instructed to not consume these at least 60 min prior to their training. This was to avoid any beneficial effect of citrate on performance (Potteiger et al., 1996, Oöpik et al., 2003, Kowalchuk et al., 1989).

3.3 Testing protocols

3.3.1 Testing conditions

In order to minimise within-participant variation, each participant reported for testing at the same time of the day for each trial after a fasting period ranging from 4 to 12 hours. Participants were also instructed to refrain from alcohol and caffeine for 24 hours prior to each trial and to be adequately hydrated. They also wore similar clothing and the same footwear on each testing occasion. The SRM ergometer was set in the same position in all the tests by recording the individual settings at each adjustment point (Fig. 3.8). Room temperature for testing was recorded and used as part of calculation in spiroergometry analysis. Participants were also asked to refrain from training on the test days and most included the test as their training session for the day. This enabled maintenance of a high level of protocol adherence without demanding unreasonable lapses in training schedules.

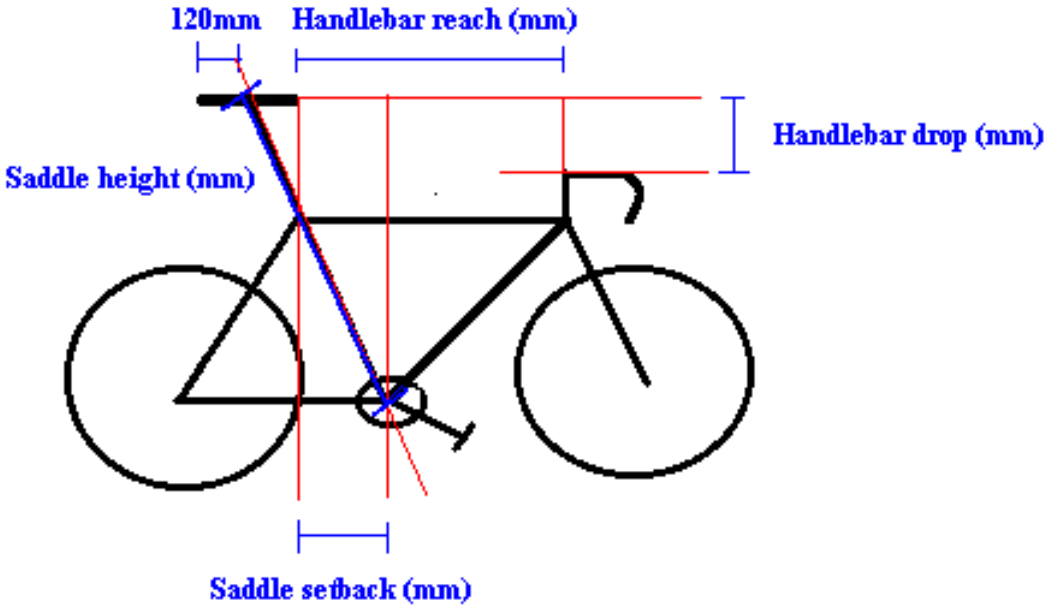


Fig. 3.8 Schematic representation of the cycle ergometer set-up measurements for each participant (Wooles et. Al., 2003).

3.3.2 $W_{peak\ (aerobic)}$ Test (Study 2 and 3)

A peak min-power test according to the ramp test protocol defined by the British Cycling Federation (BCF) v1.6 (Wooles et al., 2003) was used in study 2. This test was performed on a stationary electromagnetically braked cycle ergometer (SRM ergometer; Schoberer Rad Messtechnik, Jülich, Germany). After a 5-15 min warm up at workloads selected by the participants the test was commenced. The gear setting was standardised for all athletes at 9. The starting wattage was determined by the body mass of the athlete (BCF guidelines, Wooles et al., 2003) and ramp rate was set at 20 W/min until volitional exhaustion. Gas exchange data was collected throughout the test, in addition to the power profile and HR. All variables were averaged every 10 sec and $W_{peak\ (aerobic)}$ was determined as the average power on the last completed stage.

3.3.3 Time Trials (TT)

All tests were carried out with at least three days between each testing both the placebo and the intervention phases of the trial. The tests began with the riders emptying their bladder, body mass being recorded and used to determine relative oxygen consumption in the gas exchange data collection. Resting blood samples were then collected before a self selected warm up protocol was allowed for all the participants. There were no restrictions with respect to time and intensity of the warm up phase. When the participants were ready to start they assumed their riding position, the computer screens with all measurement displays of power output, HR, gas exchange and time elapsed were turned so that the riders had no view of them. In addition the power control monitor on the cycle ergometer was also shielded.

However, they were allowed to know any one of the following parameters: cadence, HR or time elapsed during the test. And the same one parameter was then disclosed to them for all the subsequent time-trials.

The tests were performed on the 'open ended' setting of the SRM ergometer with a self-selected cadence. Four TT were conducted in total - a 10 mile baseline test (10TTB), a 25 mile baseline test (25TTB) and these were then repeated following the intervention, a 10 mile post-calcium test (10TTC) and 25 mile post-calcium test (25TTC). Gas exchange data was collected throughout; capillary blood samples were taken every 5 min for the 10 TT and 20 min for the 25 TT for analysis of lactate, glucose, and NEFA. Data was averaged at 5 min interval for the 10 TT and 20 min intervals for the 25TT. Water was allowed *ad libitum* throughout the tests. No verbal or other forms of encouragement were given during any tests.

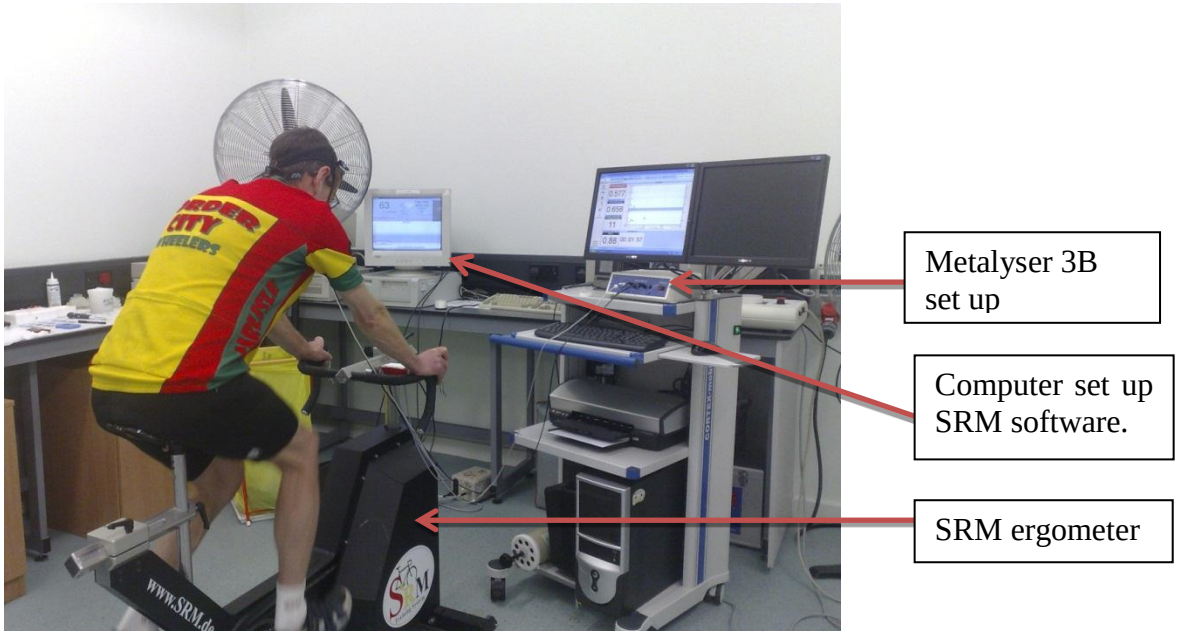


Plate 3.1 Laboratory set up for study 2 and 3

3.4 Justification for chosen Methodology

3.4.1 Seven day diet diary (unweighed)

Recorded food diaries are usually used for individual dietary data collection (Morgan et al., 1978). A dietary record of seven consecutive days or 20 consecutive meals has been reported as the least amount of time needed for an accurate report of the individual's eating habits (Leverton and Marsh, 1939). The seven day diary is considered to be better than other forms of recall such as 24 hour diet recall or even shorter periods of recorded diaries (Young, 1981). However, it has many confounded issues that must be highlighted in order to make a realistic judgement on the recordings of the participants in this study. Content validity has been fervently discussed in the literature on recording dietary patterns. The Hawthorne effect of recording food has been a particular bane for most scientists. In order to study "actual" intake (observed without the knowledge of the participant) against that of "recorded" intake, the persistent issue of ethical considerations may in effect create barriers in maintaining true validity. In fact Young (1981) argues that "true validity" cannot be measured in such scenarios. Moreover, it is imperative to remember that a diet record is not a true estimation of nutrient status (Young, 1981). It can however be assumed that if dietary intake is adequate then the person is also likely to be sufficient in nutrients (Bingham et al., 1997).

Not much has been published on athlete habits of recording diet history compared to the general population. There are a few studies reporting the differences in nutrient intakes between these populations (Grandjean, 1989) but not on how they differ in the reporting of these intakes. Past experience of the author of working with athletes both elite and recreational has led to the assumption that under reporting is less of a

concern in this population. The key difference being that, athletes are motivated to understand their nutrition and are generally motivated by any aspect of their habits that may achieve better performance. When the participants in these studies were explained how the accuracy of their reporting affected the accuracy of the analysis, they were further motivated to provide accurate reports of their eating patterns. But as discussed above the limitations of a dietary recall means that the data presented in this thesis is likely to be inflicted by such errors.

3.4.2 Quantification of training via TRIMP

There are several methods described in the literature for quantifying training, such as MET equivalents (Ainsworth et al., 2000), lactate threshold zones (Janssen, 2001) and various other forms of multiplicative factors that identify training intensity and volume such as those based on RPE and HR. Physical activity logs are considered to be quite suitable over periods of several days and weeks over other methods such as observational and physiological techniques (Hopkins, 1998)**.** Hopkins (1998) also warns against collecting too much detail in order to not overwhelm both the participant and the researcher. The most prevalent model of quantifying training in sports is the Fitness-Fatigue model championed by Banister et al. (1975). This model uses HR to quantify training (input) in order to assess performance (output). But the issue with the Banister et al. (1975) model is that it does not take into account intensity of training which may have a huge impact on performance and indeed recovery. Thus the HR zone multiplier suggested by Foster et al. (2001) seemed more appropriate in this instance where the participants were involved in a variety of different training protocols that may have impacted on their performance during the

laboratory testing four weeks apart. But the limitation of this method is that it relies on HR which has been shown to have great intra-variability (Aubert et al., 2003) and also it has been shown that the TRIMP method may have some limitations in terms of quantifying weight training and other such related activities (Foster et al., 2001). But since most the participants at least in study 2 and 3 performed a majority of their training as a variant of a cycling based session this did not pose a great problem. Moreover, most also had HR monitors in addition some had detailed power output and other training date available to hand. Those who did not posses a HR monitor were loaned one from the laboratory facilities. Overall, for the purpose of this investigation, in order to log a mean training pattern of the participants and not interfere with any prescriptive training, the TRIMP method to quantify their training was deemed valid and suiTable.

3.4.3 Time Trials

To evaluate the performance ability of endurance athletes, it is necessary to use laboratory based tests that are reliable, valid and sensitive to small changes in the athlete's abilities to perform a particular task (Hopkins et al., 1999). This is particularly important when performing repeated measures and when laboratory data needs to be applied to the field to predict actual performance. To investigate the relationship between physiological variables measured during a laboratory test with field based cycling performance, studies have used time to complete time trials as a criterion measure (Coyle et al., 1991, Hawley and Noakes, 1992, Miller and Manfredi, 1987, Palmer et al., 1996) which has shown good correlations with actual performance in race times. Coyle et al. (1991) have demonstrated a close association

between performance during an actual 40 km time trial and one hour laboratory test, indicating that use of such protocols simulate actual time trials reasonably well and therefore these tests in the laboratory generally reflect the demands during a race event. This is not surprising considering that in contrast to most laboratory based tests; TTs present a unique experience during which the athlete's power output (i.e. exercise intensity) is freely chosen thus closely simulating race conditions.

3.4.4 Gross Efficiency (GE) as a measure to study metabolic changes due to calcium supplementation

One of the main variables that will be used to evaluate the ergogenic effect of supplemental calcium is the surrogate measure of the chemomechanical property, efficiency. The basic definition of gross efficiency (GE) is the ratio of work done during the specific activity to the total energy expended and expressed as a percentage (Sidossis et al., 1992). Muscular efficiency in studies 2 and 3 has been expressed in the form of GE from whole body measurements of $\dot{V}O_2$ during the TT. It is important to discuss the assumptions and the limitations of this approach. GE estimates were dependant on the validity of estimating energy expenditure from whole body $\dot{V}O_2$. Although whole body respiratory gas exchange can be measured very reliably, it is difficult to determine what percentage of the TEE contributes to actual physical work accomplished as opposed to the energy expended for other bodily processes including stabilization and limb movement. Grasser and Brooks (1975) and then later Stainbsy et al. (1980) suggested baseline subtractions to determine net efficiency. This is to prevent the essentially linear relationship between work rate and energy expenditure to make it appear that efficiency increases

with work rate. Therefore the main aim of baseline subtraction is to establish a measure that refers to the efficiency of muscle contraction *in-vivo*. Net efficiency (NE) where the baseline is the energy expended at rest and work efficiency (WE) where the baseline is the energy cost of unloaded cycling (typically 5 kJ/min) (Stainbsy et al., 1980). Ettema and Lorås (2009) argues that while this procedure may be relatively simple in engineering, it is not so in biology because of the many interactions and interdependences between physiological systems. By using baseline subtractions one implicitly assumes that the processes related to resting metabolism are independent, constant and essentially isolated from the processes of doing work (Moseley and Jeukendrup, 2001). It may be incorrect to assume that the energy cost of baseline processes is unaffected by the work rate during exercise (Ettema and Lorås, 2009). One can argue that is precisely why GE increases with work rate because of the diminishing effect of offset (baseline) metabolism.

Another approach to quantify muscular efficiency of work rate that could be applied is to divide the total work rate into internal and external work (WE). Internal work is the work necessary to move the body segments relative to the body's centre of mass, i.e. changes in the relative kinetic and potential energy of the segments. The cost of body segment movement energy changes is generally measured by determining energy cost for zero work rate (Hintzy-Cloutier et al., 2003). By measuring energy cost of unloaded movement (graded work rate protocol at the same cadence in case of cycling), the assumption is that all internal work is dissipated into heat because the circumstances do not allow for external work. But studies measuring metabolic cost of zero work rates have shown that moving lower extremities passively, metabolic rate increases significantly (Nobrega et al., 1994). Other studies report that muscle activity in unloaded cycling is extremely low and can hardly account for the

total increase in metabolic rate 240-450 W at zero external work at 60-120 rpm (Ettema and Lorås, 2009; Siddossis et al., 1992). This may indicate that other processes than simply the active limb movements also affect metabolic rate. Some of the possible contributing factors suggested in the literature range from coordination challenges (Neptune and Herzog, 1999), enhanced heart and ventilation rates (Aaron et al., 1992) and muscle activity (Bell et al., 2003). Since the metabolic rate at unloaded cycling is the sum of resting metabolic rate and the metabolic rate required for doing internal work, and as seen from the above arguments, these measurements neglect the inter-dependence in the physiological system for measuring muscular efficiency in whole body movements. Even though it is generally agreed that GE is a poor measure of the efficiency of muscular work (Moseley et al., 2004), it has been suggested the GE is a better measure of whole body efficiency (Ettema and Lorås, 2009).

Since calcium changes in the cellular compartments are closely linked to changes in ionic concentrations of blood calcium levels. Changes in dietary calcium may have an effect on various tissue concentrations of calcium such as adipose tissue (Zemel, 1998), muscle tissue (Russell et al., 1984) and metabolic changes in both these tissues may affect performance and efficiency via changes in physiological environment that regulates homeostasis. So if any efficiency gains are achieved through the "non-performance" related changes in efficiency of use of substrates or changes in flux of metabolites in the muscle cells may be better detected via measurements of GE. Especially as variation of GE is half that of DE, suggesting that smaller changes in efficiency may be better detected in GE than DE (Moseley and Jeukendrup, 2001). Although, Coyle et al. (1991) has suggested that delta efficiency (reciprocal slope of the linear relationship between energy expenditure

and work rate) as a more valid estimate of muscular efficiency, another study (Moseley and Jeukendrup, 2001) has showed this to be less reliable than GE. Therefore the use GE can be used as a close surrogate measure in quantifying the various systems that may have been involved in the metabolic processes that could be affected by the interaction of supplementary calcium and the ensuing metabolic changes. In addition, measures have been taken to further accommodate the reliability of the data by studying closely matched well-trained participants.

GE was calculated by dividing the external power output by the metabolic power output (Cooke, 2004), where –

External Power output (W) = Force (N) $\times$ Distance (m) / Time (s) and

Metabolic work-rate (W) = $(\dot{V}O_2$ l/min) $\times$ 348.8

$$\text{Thus \% of GE} = \frac{[(\text{Force X Distance}) / \text{Time}]}{\dot{V}O_2\,(\text{l/min}) \times 348.8} \times 100$$

3.4.5 Validity of substrate oxidation rates calculated from RER measurements.

Estimation of energy expenditure by indirect calorimetry is based on the assumption that heat production within the body occurs by aerobic processes. Since the products of anaerobic metabolism have to be oxidised after a few seconds (e.g. lactate) this assumption is not invalid. During exercise however, some assumptions on heat production need to applied in the light that all energy produced by oxidation of substrates might not be released as heat due to some storage as potential energy in order for external work to be maintained (Frayn and Macdonald, 1997). Also energy expenditure calculated as a measure of constant rate of energy release per litre of

oxygen consumption has a larger proportion of error compared to energy production calculated as a sum of different substrates used. Rate of energy release from different macronutrients is different. For example protein is 19.48 kJ/l of O_2 and 21.12 kJ/l of O_2 for carbohydrates. Therefore an intermediate value of 20.3 kJ/l of O_2 can produce an error up to 4% (Frayn and Macdonald, 1997). Thus, calculations based on energy equivalents of the oxygen consumed to the carbon dioxide produced for macronutrients seem more appropriate. The smaller variation calculated in energy released during the oxidation of the same substrates such as glucose (20.84 kJ/l of O_2) than of starch or glycogen (21.12 kJ/l of O_2) leads to an error of $\leq$ 1% (Frayn and Macdonald, 1997). Frayn (1983) has provided stoichiometric equations for calculations of net substrate oxidation rates from gas exchange measurements. These are -

$$\text{CHO oxidation} = 4.55\ \dot{V}CO_2 - 3.21\ \dot{V}O_2 - 2.87\ n$$

$$\text{Fat oxidation} = 1.67\ \dot{V}O_2 - 1.67\ \dot{V}CO_2 - 1.92\ n$$

Where n = grams of urinary nitrogen per min

This method has several limitations that should be considered in the interpretation of the data. First the gas exchange measurements are a reflection of the different simultaneous biochemical processes which may have offsetting results on the calculation of substrate oxidation rates. For example, both gluconeogenesis from amino acids and ketogenesis produce a respiratory quotient below 0.7 (Frayn, 1983). These circumstances will result in the underestimation of carbohydrate oxidation.

Second, urinary nitrogen excretion during exercise is probably not an accurate reflection of protein oxidation because exercise alters renal perfusion. This may

change the renal clearance rate of urea. However because the contribution of protein oxidation to total energy metabolism is small in exercise, an error in the quantification of protein oxidation will not significantly affect the calculation of carbohydrate and fat oxidation. The substrate oxidation rates were calculated assuming that nitrogen excretion would remain constant during rest and exercise (Carraro et al., 1990). As suggested in the literature the values of nitrogen excretion for the purpose of calculations was assumed to be maintained at 135 μg/kg/min (Romijn et al., 2000).

Third, indirect calorimetry relies on the assumption that pulmonary gas exchange reflects the rate of oxygen consumption and carbon dioxide production at the tissue level (Jansson, 1982). This is probably true for oxygen consumption because there are no large stores of oxygen in the body. However carbon dioxide production as measured by analysis of expired air is a reliable estimate of tissue carbon dioxide production only if the large bicarbonate pool is stable (Sutton et al., 1981). But exercise above the ventilatory and blood lactate thresholds result in a compensatory depletion of the bicarbonate pool the extent of which depends on the intensity and duration of exercise in that case carbon dioxide production as estimated by measurements of expired air is composed of both tissue carbon dioxide production and carbon dioxide derived from the bicarbonate pool (Lamb and Gisolfi, 1988). This will result in erroneously higher values of carbohydrate oxidation as calculated by indirect calorimetry. Even at exercise levels below the lactate threshold bicarbonate kinetics are markedly altered (Heigenhauser and Jones 1991, Sutton et al., 1981). The margin of error would be higher at higher exercise intensities of exercise.

And thus even though it is not possible to prove conclusively the share of each substrate in energy production during rest and exercise using respiratory gas exchange measurements, studies using stable isotopes have provided some validity on the reliability of this method (Romijn et al., 2000, Phillips et al., 1996, Schoeller, 1988).

3.5 Statistical analysis

Descriptive data and all related analysis of significance were generated using PASW (Predictive Analytics SoftWare) Statistics Version 17 (SPSS Inc., Illinois, USA). Significance level was set at 95% confidence intervals (CI) ($p < 0.05$). Data are represented as measures of centrality and spread (mean ± standard deviation (SD)). Uncertainty in the estimates of effects is expressed as 90% confidence intervals (CI).

Mean differences were compared using paired t-tests and differences of means between trials and within trials were analysed using factorial repeated measures design. Mauchly's sphericity test was conducted to determine if the assumption for sphericity of the data was met. If this was violated ($p < 0.05$) the F value was corrected by reporting significance of the Greenhouse-Geisser test. Main effect for time, main effect for trials and interaction between trials*time was used to detect statistical significance at each time point and across each trial respectively. The Bonferroni post-hoc test was used to report where these statistical differences were observed. Where appropriate, Pearson's correlation (*r*) was performed to compare the relationships between variables. Magnitudes of inferences were calculated using Cohen's *d* and where appropriate reported along with p values.

The degree of heteroscedasticity of the incremental test data was determined by calculating Pearson's product moment correlation coefficients (R^2) between the difference and the average value of the physiological variables in the incremental tests.

Bland-Altman plots were produced to determine the repeatability of gas exchange and power output data. The reporting of the Limits of Agreement (LoA) is represented by systematic bias (mean difference of two measurements) and random error (standard deviations of the mean difference). LoA were calculated and plots produced with 95% CI (1.96 x SD) only if the data satisfied conditions that the test-retest differences among participants were normally distributed, and if the test and retest means were not significantly different and if there was no significant relationship between test-retest differences and test-retest means (Lamb, 1998).

All reliability statistics are presented as %CV and/or change in mean, typical error (TE) and Pearson's product moment correlation coefficient with 95%LoA where appropriate.

CHAPTER 4

EFFECTS OF CALCIUM SUPPLEMENTATION ON SUBSTRATE METABOLISM DURING SUBMAXIMAL EXERCISE

4.1 Introduction

Epidemiological data suggests a positive relationship between increased calcium intake and decreased fat and total body mass in healthy people (Barr, 2003; Davies et al., 2000). High calcium diets have shown to markedly inhibits lipogenesis (Jones et al., 1998; Kim et al., 1996; Xue et al., 1998), accelerate lipolysis (Zemel et al., 2000), increase thermogenesis and suppress fat accretion and weight gain in identical caloric intakes (Shi et al., 2001a; Zemel et al., 2000).

Recent data from various studies revealed that increased PTH resulting from low dietary calcium is associated with increased fat mass in healthy young women (Gunther et al., 2006; Gunther et al., 2005). Xue et al. (1998) reported the link between increased $_{i}[Ca^{2+}]$ and lipogenesis in rat adipose tissue. They suggested that the anti-lipolytic effect of $_{i}[Ca^{2+}]$ may be mediated by the activation of phosphodiesterase 3B (PDE) leading to a decrease in cAMP and hormone sensitive lipase (HSL) phosphorylation and consequently inhibition of lipolysis (Xue et al., 2001).

This mechanism is well established *in-vitro*, although the influence of calcium on lipolysis during exercise in humans is yet to be investigated. A positive correlation between plasma calcium levels and NEFA oxidation in endurance-trained athletes has been reported (Men'shikov, 2004). There are no studies on the effects of long-term calcium supplementation on substrate metabolism during sub-maximal long duration exercise. This first study aims to identify if the *in-vitro* model can be applied in the free living population with co-relatable inferences such as changes in body composition. In order to explore the effects of calcium supplementation during exercise in humans, it is important to study these effects on a heterogeneous athletic population first.

Therefore, the aim of this experimental trial was to investigate the effects of calcium supplementation on substrate metabolism during sub-maximal exercise. These investigations were conducted on a mixed gender population of varying training status. The study also aims to investigate the effect of calcium supplementation on body composition changes of the athletes.

4.1.1 Hypotheses

i. It is hypothesised that calcium supplementation of 1000 mg/d for four weeks results in increased fat oxidation during sub-maximal exercise.

ii. It is also hypothesised that calcium supplementation of 1000 mg/d for four weeks reduces body fat mass of recreationally trained individuals.

4.2 Methods

4. 2.1 Participants

Nine moderately trained (trained for 1-2 hours/day for 4-6 days/week) participants were recruited in this study. Inclusion criteria as described in section 3.1 were met. These were recruited from a cohort of sports science students and staff at the University of Central Lancashire. All participants were physically active and were trained at recreational level. The mean (± SD) physical characteristics of the participants are given in table 4.1.

Table 4.1 Mean (± SD) physical characteristics of participants (n = 9) in study 1

	Age (years)	Weight (Kg)	Height (cm)	$\dot{V}O_{2peak}$ (l/min)	$\dot{V}O_{2peak}$ (ml/kg/min)	$W_{peak(aerobic)}$ (W)
Mean (SD)	40.56 ± 9.51	71.33 ± 10.81	171.8 ± 7.50	3.17 ± 1.35	48.10 ± 14.44	314 ± 94

4.2.2 Equipment (not described in Chapter 3)

4.2.2a Lode Excalibur Sport cycle ergometer

A stationary electromagnetically braked cycle ergometer (Lode Excalibur Sport; Lode Medical Technology, Groningen, The Netherlands) was used. The ergometer is controlled by an external unit allowing for adjustments of work load, rpm, time and distance (plate 4.1). The workload can be controlled in isokinetic, hyperbolic and linear modes. It uses the same strain gauge principle as described in section 3.2.1a

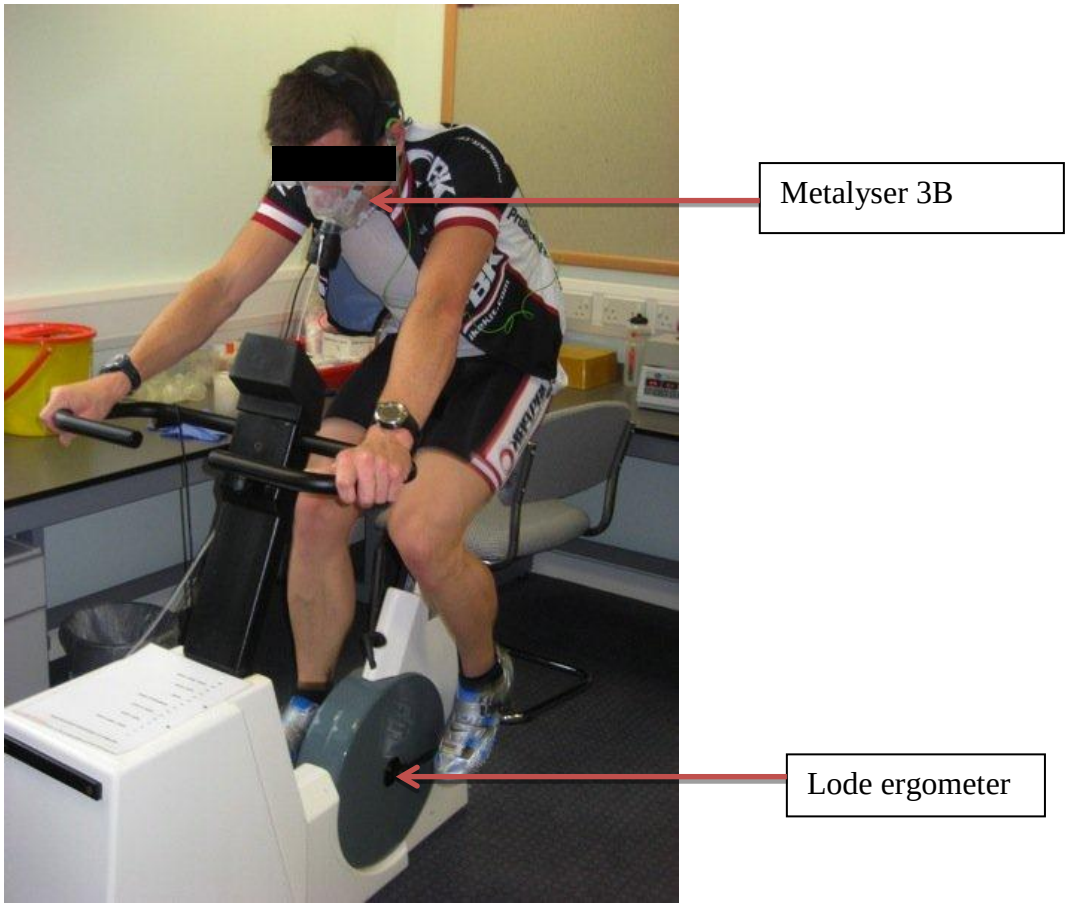


Plate 4.1 The Lode setup

4.2.2b Skinfold measurements and body composition estimation

Skinfold thickness was measured to the nearest 0.5 mm with a calibrated skinfold calliper (Harpenden, Body care, Kenilworth, UK) at seven anatomical sites by the same trained technician (author). The calliper exerts a constant pressure at varying openings of the jaws. The width of the opening is read off on a scale incorporated in the apparatus. The seven sites measured were pectoral, mid-axilla, abdominal, suprailium, subscapular, triceps and mid-thigh. All measurements were taken on the participant's right side. Percentage body fat (%fat) values were estimated by two different methods. The seven skinfold site measurements were used to estimate body density (Jackson and Pollock, 1978; Jackson et al., 1980), and %fat

subsequently calculated by the Siri equation (Siri, 1956). Muscle mass was estimated using the skinfold-circumference model from *in-vivo* derived equations in Lee et al. (2000).

Reliability of the measurements was established by conducting 3 trials on the seven sites mentioned above (n = 11) on consecutive days. Inter-test reliability results are presented in table 4.2 by calculating the difference of mean between trial 1 and trial 2.

Table 4.2 Change in mean, typical error and correlation coefficient of skinfold measurement. Units ± SD (95% CI upper to lower limits)

	Change in mean	Typical error	Correlation coefficient Pearson's (R^2)
PECTORAL	**0.26 ± 0.95** (-0.38 to 0.9)	**0.69** (0.48 to 1.21)	**0.99** (0.97 to 1.00)
AXILLA	**0.64 ± 1.68** (-0.49 to 1.76)	**1.21** (0.85 to 2.13)	**0.98** (0.93 to 1.00)
ABDOMINAL	**0.23 ± 2.66** (-1.56 to 2.02)	**1.93** (1.35 to 3.39)	**0.98** (0.94 to 1.00)
SUPRAILIUM	**-0.53 ± 1.61** (-1.61 to 0.55)	**1.17** (0.81 to 2.26)	**0.99** (0.94 to 1.00)
SUBSCAPULAR	**0.73 ± 2.29** (-0.81 to 2.26)	**1.66** (1.16 to 2.91)	**0.95** (0.82 to 0.99)
TRICEPS	**-0.20 ± 1.18** (-0.99 to 0.59)	**0.85** (0.60 to 1.50)	**0.98** (0.94 to 1.00)
MIDTHIGH	**-0.26± 1.20** (-1.07 to 0.54)	**0.87** (0.61 to 1.53)	**0.99** (0.96 to 1.00)

4.2.3 Experimental design

The study was designed as a randomised, double blind, placebo controlled crossover intervention. Participants were assigned to either a placebo (Pl) or calcium intervention (Ca) group at the start of the study, with a subsequent crossover following a four week 'wash out' period. Randomisation was achieved by placing the first participant enrolled in the trial A group as first order, followed by the second participant in the trial B group as first order. This was repeated until optimal recruitment number was achieved. The researcher (author) was not privy to the randomisation. This allowed for even number recruitment in each group from the start of the experiment and enrolment to the trial was on a continuous rolling basis.

The formulation procedure for both the calcium and the placebo capsules is described in section 3.2.7. The timeline of the supplementation schedule is depicted in fig 4.1. Each phase of the intervention lasted for four weeks, with baseline measurements and testing performed at the start and finish of both phases of the intervention.

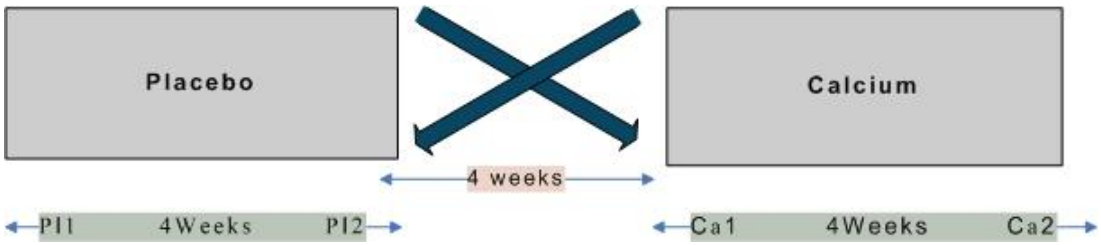


Fig. 4.1 Schematic representation of the experimental design in study 1

4.2.4 Testing protocols

4.2.4a $W_{peak\ (aerobic)}$ test

All participants underwent a $W_{peak\ (aerobic)}$ test one at a time. Participants reported to the laboratory in a post prandial state of at least one meal consumed about 60 to 90 min prior to testing. An incremental continuous protocol was used to elicit the peak volume of oxygen uptake ($\dot{V}O_{2peak}$) and peak aerobic power production ($W_{peak\ (aerobic)}$). After a 5-10 min warm up on a self-selected load and cadence the participants performed a continuous incremental test on a cycle ergometer (Lode Excalibur Sport; Lode Medical Technology, Groningen, The Netherlands) starting at a 180 W and increasing by 5 W every 10 sec until volitional exhaustion. The participants pedalled at a consistent self-selected cadence (55-110 rpm). The seat height and handlebar position were adjusted to each participant's personal preference. This particular protocol was chosen in study 1 as the participant demographics varied widely (table 4.1). Even though it did not allow for steady state measurements of gas exchange at every submaximal stage it was better suited to those who were not used to maximal tests and/or very high intensity protocols as it allowed for valid determination of peak oxygen uptake values (Astrand and Rodahl, 1986). Gas exchange data was recorded throughout using the Cortex Metalyzer 3B laboratory Breath-by-Breath spiroergometry System and blood indices for lactate concentrations was measured as per section 3.2.4b at rest, beginning of the incremental test, end of every 2 min and the last measurement at test termination. $\dot{V}O_{2peak}$ was accepted as the highest observed value provided that there was $\leq$ 2 ml /kg/min increase in $\dot{V}O_2$ uptake with increasing workload or the participant

volitionally terminated the test. All gas exchange data was averaged for a 10 sec time period which was then used to calculate $\dot{V}O_{2peak}$ and end wattage (W_{peak}) at the last completed min was averaged and recorded. The PO_{peak} determined at the last completed stage before accepting the $\dot{V}O_{2peak}$ values was then used to set the 50% maximal intensity load.

4.2.4b Steady state 50% $W_{peak\ (aerobic)}$ protocol

A 60 min 50% W_{peak} protocol was used to study substrate metabolism during a sub-maximal exercise. This protocol was selected to elicit maximum fat oxidation rates (Achten et al., 2002). Participants adhered to conditions described in section 3.3.1 before commencing the test on a cycle ergometer (Lode Excalibur Sport; Lode Medical Technology, Groningen, The Netherlands). The set-up of the ergometer was kept same with regards to saddle height and handlebar angles as in the incremental test. A 5 min warm up was allowed before commencing the actual test. The cycle ergometer was set at a wattage that corresponded to 50% W_{peak} values from the incremental test. HR was monitored throughout to ensure that the required intensity was maintained by cross referencing those values with that of the incremental tests and predicted HR values.

Gas exchange (Cortex Metalyzer 3B laboratory Breath-by-Breath spiroergometry System Cortex Biophysik GMBH, Version ML3B 2.1) data was collected throughout. All data was averaged at 60 sec intervals for analysis. One venous blood sample as described in section 3.2.4a was taken prior to the start of the test for analysis of iPTH and capillary blood samples taken as described in section 3.2.4b for analysis of lactate, total plasma calcium, plasma triacylglycerides, plasma glucose

and NEFA every 20 min just before recording an RPE score till test termination. Water was allowed *ad-libitum* throughout the tests. No verbal or other forms of encouragement were given during the tests.

4.2.4c Dietary and physical activity records

All participants were asked to keep a seven day diet diary and physical activity log as described in section 3.2.6a during any typical week of the intervention and were asked to repeat the same in the following intervention. The timeline for testing variables are represented in a schematic diagram in fig 4.2.

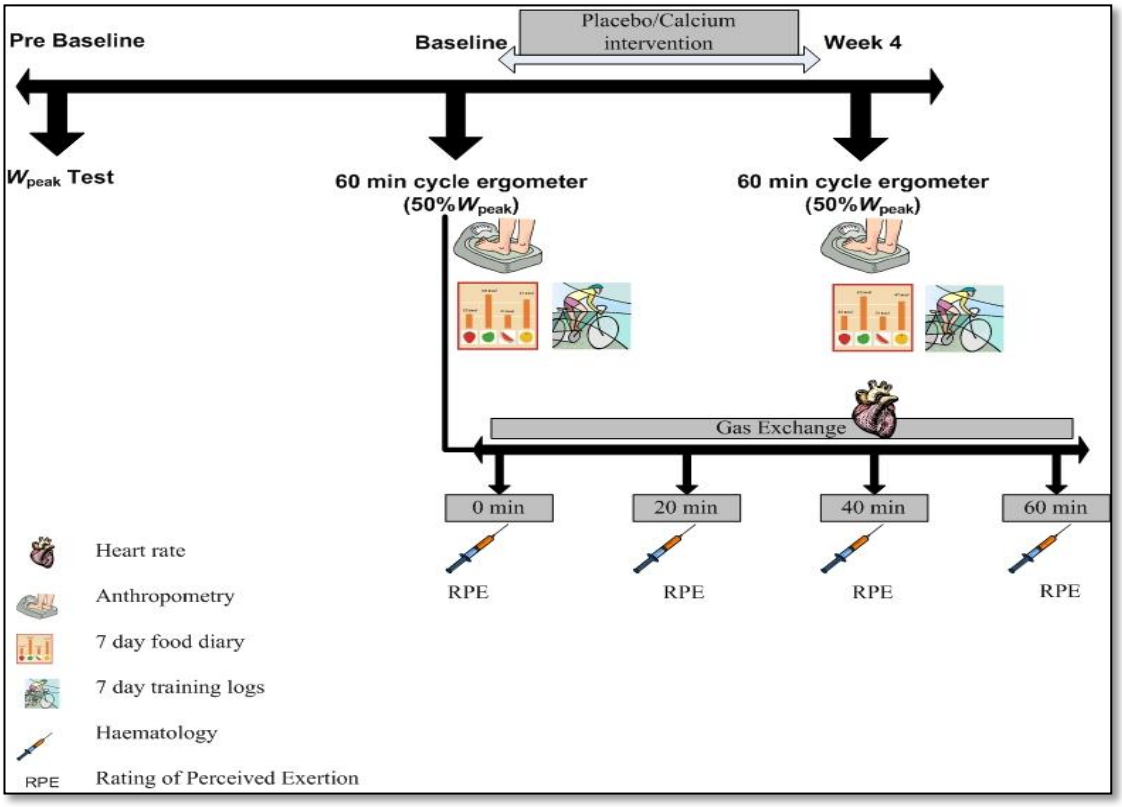


Fig. 4.2 Schematic representation of the experimental protocol within each intervention

4.2.4d Statistical analysis

All data was analysed and statistical analysis conducted as described in section 3.5.

4.3 Results

4.3.1 Anthropometry

Mean change in anthropometric values from the placebo trial are presented in table 4.3. There was no statistically significant change in total body mass ($p = 0.12$), lean mass ($p = 0.33$) or fat mass ($p = 0.10$) after the Pl2.

Table 4.3 Mean (± SD) anthropometric changes in participants (n = 9) of study 1 in the placebo trial before (Pl1) and after (Pl2) four weeks

n = 9	Pl1 (mean ± SD)	Pl2 (mean ± SD)	Difference; ± 90%CI	*(d)*
Total Mass (kg)	71.74 ± 10.8	71.50 ± 11.35	-0.24; ± 0.33	-0.02
BMI (kg/m^2)	24.25 ± 2.43	24.17 ± 2.40	-0.08; ± 0.03	-0.01
Fat Mass (kg)	18.00 ± 5.90	18.10 ± 5.02	0.10; ± 0.91	0.02
Lean Mass (kg)	53.74 ± 9.84	53.40 ± 9.87	-0.34; ± 0.03	-0.03

Mean anthropomentric changes after the calcium intervention are presented in table 4.4. After the Ca2 trial there was a statistically significant decrease in fat mass ($p = 0.02$). There was a statistically non-significant loss of mean total body mass ($p = 0.09$) and a statistically non-significant increase in lean mass ($p = 0.08$) after four weeks of calcium supplementation.

Table 4.4 **Mean (± SD) anthropometric changes in participants of study 1 in the calcium trial before (Ca1) and after (Ca2) four weeks**

n = 9	Ca1 (mean ± SD)	Ca2 (mean ± SD)	Difference; ± 90%CI	*(d)*
Total Mass (kg)	73.72 ± 11.3	73.40 ± 10.60	-0.32; ± 0.75†	-0.02
BMI (kg/m^2)	24.92 ± 2.38	24.81 ± 2.13	-0.11; ± 0.21	-0.05
Fat Mass (kg)	18.59 ± 4.80	18.00 ± 5.21	-0.59; ± 0.42*	-0.20
Lean Mass (kg)	55.13 ± 8.84	55.40 ± 9.87	0.27; ± 1.07†	0.04

***significant at p < 0.05 †approaching significance at p ≤ 0.10**

Further analysis revealed that the changes in anthropometric values showed different trends in recreationally trained (n = 4) sub-group compared to the well-trained (n = 5). The mean anthropometric changes during the placebo trial in recreational participants are presented in table 4.5. Total mass (p = 0.07) and fat mass (p = 0.08) increased during the four weeks; however these changes were not statistically significant.

Table 4.5 **Mean (± SD) anthropometric changes in recreationally trained participants of study 1 before (Pl1) and after (Pl2) placebo intervention for four weeks**

n = 4	Pl1 (mean ± SD)	Pl2 (mean ± SD)	Difference; ± 90%CI	*(d)*
Total Mass (kg)	64.34 ± 8.03	65.37 ± 7.41	1.03; ± 0.62†	0.13
BMI (kg/m^2)	23.35 ± 2.33	23.72 ± 2.08	0.37; ± 0.25	0.08
Fat Mass (kg)	20.17 ± 4.05	21.44 ± 4.76	1.27; ± 0.71†	0.23
Lean Mass (kg)	44.17 ± 5.44	43.93 ± 2.63	-0.24; ± 2.82	0.03

†approaching significance at p ≤ 0.10

The mean changes in anthropometric values after the calcium trial in the recreationally trained athletes are presented in table 4.6. Mean total mass (p = 0.27) and mean BMI (p = 0.17) decreased non-significantly along with lean mass (p = 0.08) but fat mass showed a trend to increase (p = 0.07).

Table 4.6 **Mean (± SD) anthropometric changes in recreationally trained participants of study 1 before (Ca1) and after (Ca2) calcium intervention for four weeks**

n = 4	Ca1 (mean ± SD)	Ca2 (mean ± SD)	Difference; ± 90%CI	*(d)*
Total Mass (kg)	64.01 ± 8.13	63.52 ± 8.26	-0.49; ± 0.13	-0.12
BMI (kg/m^2)	23.23 ± 2.18	23.05 ± 2.22	-0.18; ± 0.04	-0.10
Fat Mass (kg)	19.41 ± 8.04	19.73 ± 6.08	0.32; ± 1.95†	-0.06
Lean Mass (kg)	44.60 ± 4.45	43.79 ± 4.29	-0.81; ± 0.16†	-0.26

†approaching significance at p ≤ 0.10

The mean anthropometric changes in the well trained athletes during the placebo trial are presented in table 4.7. There were no statistically significant changes in the body composition in this trial. However, mean total mass (p = 0.10) and BMI (p = 0.08) increased, but did not reach statistical significance.

Table 4.7 Mean (± SD) anthropometric changes in well trained participants of study 1 before (Pl1) and after (Pl2) placebo intervention for four weeks

n = 5	Pl1 (mean ± SD)	Pl2 (mean ± SD)	Difference; ± 90%CI	*(d)*
Total Mass (kg)	77.20 ± 9.34	78.15 ± 9.61	0.95; ± 0.26†	0.10
BMI (kg/m^2)	24.92 ± 1.65	25.23 ± 2.25	0.31; ± 0.60†	0.14
Fat Mass (kg)	16.14 ± 4.26	16.45 ± 5.20	0.31; ± 0.94	0.06
Lean Mass (kg)	61.06 ± 5.31	61.70 ± 3.91	0.64; ± 1.41	0.14

†approaching significance at p ≤ 0.10

Body composition changed significantly after four weeks of calcium supplementation in the well–trained atheletes (Table 4.8). There were statistically significant changes in total body mass (p = 0.03), fat mass (p = 0.02) and BMI (p = 0.03).

Table 4.8 **Mean (± SD) anthropometric changes in well trained participants of study 1 before (Ca1) and after (Ca2) calcium intervention for four weeks**

n = 5	Ca1 (mean ± SD)	Ca2 (mean ± SD)	Difference; ± 90%CI	*(d)*
Total Mass (kg)	75.79 ± 9.26	76.83 ± 10.02	1.04; ± 0.76*	0.11
BMI (kg/m^2)	24.47 ± 2.25	24.80 ± 2.01	0.33; ± 0.24*	0.18
Fat Mass (kg)	16.20 ± 5.20	15.43 ± 4.87	-0.77; ± 0.33*	-0.14
Lean Mass (kg)	59.59 ± 3.97	61.40 ± 5.66	1.81; ± 1.69	0.31

***significant at $p < 0.05$**

4.3.2 Nutritional intake analysis

The mean variance in energy intake of the participants in both the trials during four weeks of calcium supplementation was 2.88% (Table 4.9). The mean variance in energy intake of the recreationally trained athletes was 2.66% (Table 4.10) and of the well-trained athletes was 2.65% (Table 4.11). The contribution of the various macronutrients to the energy intake is presented in Fig. 4.3 as a mean of all participants, Fig. 4.4 for the recreationally trained participants and Fig. 4.5 for the well-trained participants.

Mean calcium intake of all individual participants in the study was at least 700 mg/d (COMA, 1991). The variance between the calcium consumption in the two trials of each participant was not significant ($p = 0.12$) (appendix J).

Table 4.9 **Mean (± SD) nutrient intake in participants of study 1**

n = 9	**Trial Pl** Mean ± SD	**Trial Ca** Mean ± SD	**%CV**
Energy (MJ/d)	8.3 ± 1.5	8.3 ± 1.3	2.88
Total carbohydrates (g/d)	259 ± 72	257 ± 68	4.36
Total fat (g/d)	59 ± 8	60 ± 9	3.26
Protein (g/d)	85 ± 17	84 ± 15	2.81
Alcohol (g/d)	12 ± 9	11 ± 7	16.06
Calcium (mg/d)	770 ± 97	772 ± 114	2.40
Vitamin D (µg/d)	2.85 ± 1.34	2.80 ± 1.24	8.98

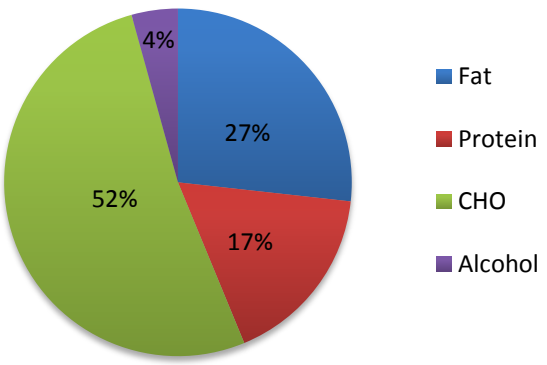


Fig. 4.3 **Mean macronutrient intake in participants of study 1 as a percentage of energy intake (n = 9)**

Table 4.10 Mean (± SD) nutrient intake in recreational participants of study 1

n = 4 (recreational)	**Trial Pl** Mean ± SD	**Trial Ca** Mean ± SD	**%CV**
Energy (MJ/d)	7.1 ± 0.94	7.2 ± 0.97	2.66
Total carbohydrates (g/d)	196 ± 50	197 ± 49	4.67
Total fat (g/d)	61 ± 5	62 ± 5	4.07
Protein (g/d)	79 ± 8	79 ± 6	2.14
Alcohol (g/d)	9 ± 6	8 ± 6	13.53
Calcium (mg/d)	765 ± 125	768 ± 163	3.82
Vitamin D (µg/d)	2.81 ± 1.78	2.73 ± 1.52	13.31

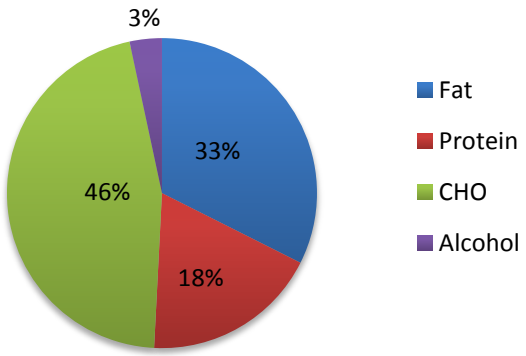


Fig. 4.4 Mean macronutrient intake in recreational participants of study 1 as a percentage of energy intake (n = 4)

Table 4.11 Mean (± SD) nutrient intake in well-trained participants of study 1

n = 5	**Trial Pl**	**Trial Ca**	**%CV**
(well-trained)	Mean ± SD	Mean ± SD	
Energy (MJ/d)	9.3 ± 1.2	9.2 ± 0.9	2.65
Total carbohydrates (g/d)	310 ± 36	305 ± 32	3.89
Total fat (g/d)	58 ± 10	59 ± 12	2.57
Protein (g/d)	90 ± 22	88 ± 19	3.41
Alcohol (g/d)	15 ± 10	13 ± 7	16.54
Calcium (mg/d)	774 ± 83	776 ± 79	0.99
Vitamin D (µg/d)	2.88 ± 1.10	2.86 ± 1.15	5.35

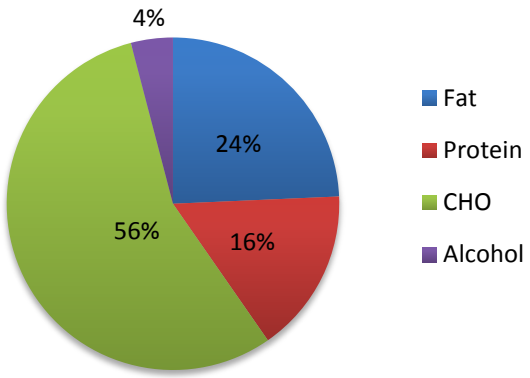


Fig. 4.5 Mean macronutrient intake in recreational participants of study 1 as a percentage of energy intake (n = 4)

4.3.3 Training logs analysis

The mean TRIMP score over seven days of all the participants in both the placebo and calcium intervention trials are presented in Fig. 4.6. The mean daily TRIMP scores over the 7 day period were not significantly different (p = 0.10).

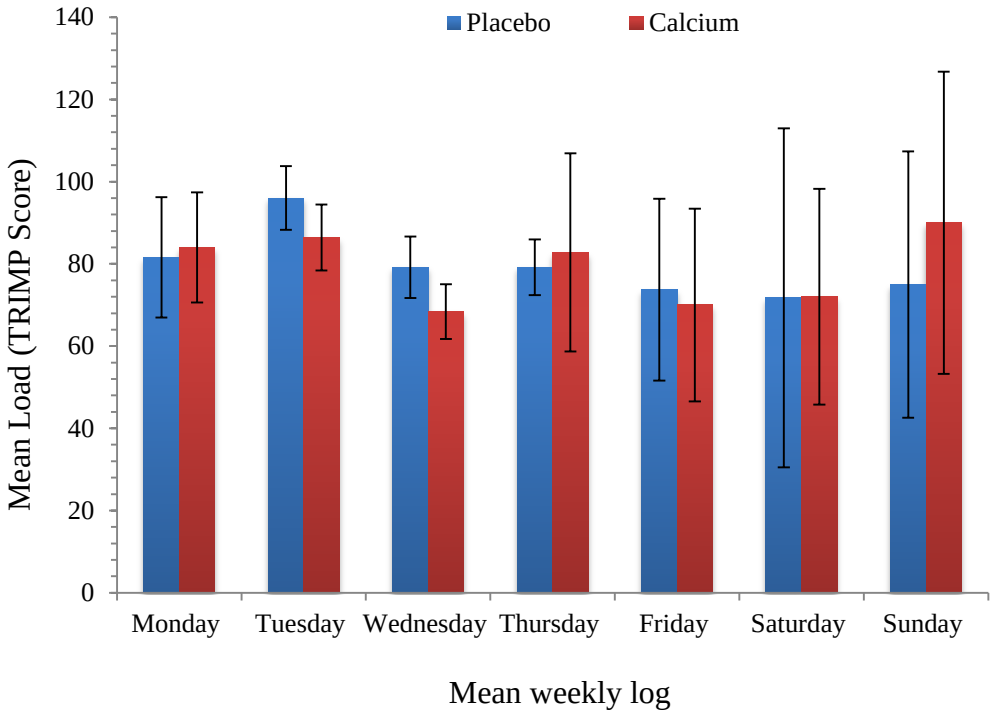


Fig. 4.6 Mean (± SD) daily load from the seven day training log of participants in both the placebo (Pl) and the calcium (Ca) intervention trials (n = 9)

The sum load of the seven days, the mean monotony and the strain of the two sets of training logs were not statistically significantly different ($p = 0.10$) and are displayed in table 4.12.

Table 4.12 Mean (± SD) quantification of training of the participants during the four weeks of supplementation in both the placebo (Pl) and the calcium (Ca) interventions

Training quantification	Trials	
	Pl	Ca
Sum weekly duration (min)	346 ± 185	340 ± 208
Mean weekly duration (min)	49 ± 26	49 ± 30
Monotony (mean daily TRIMP/SD)	10 ± 1	9 ± 2
Strain (total TRIMP · Monotony)	5486 ± 184	5014 ± 252

4.3.4 Energy expenditure

Mean total energy expenditure was significantly higher in the Ca-2 trial compared to all others (p = 0.03) (Table. 4.13).

Table 4.13 Mean (± SD) total energy expenditure of the participants in all the trials

Total energy expenditure (kJ)	Pl-1	Pl-2	Ca-1	Ca-2
(mean ± SD)	2292 ± 855	2232 ± 898	2187 ± 1003	2436 ± 1065*

*** Significant at p ≤ 0.05**

Fig.4.7 shows mean energy expenditure of all the trials over the 60 min period. There was no statistical difference between the trials at any time points.

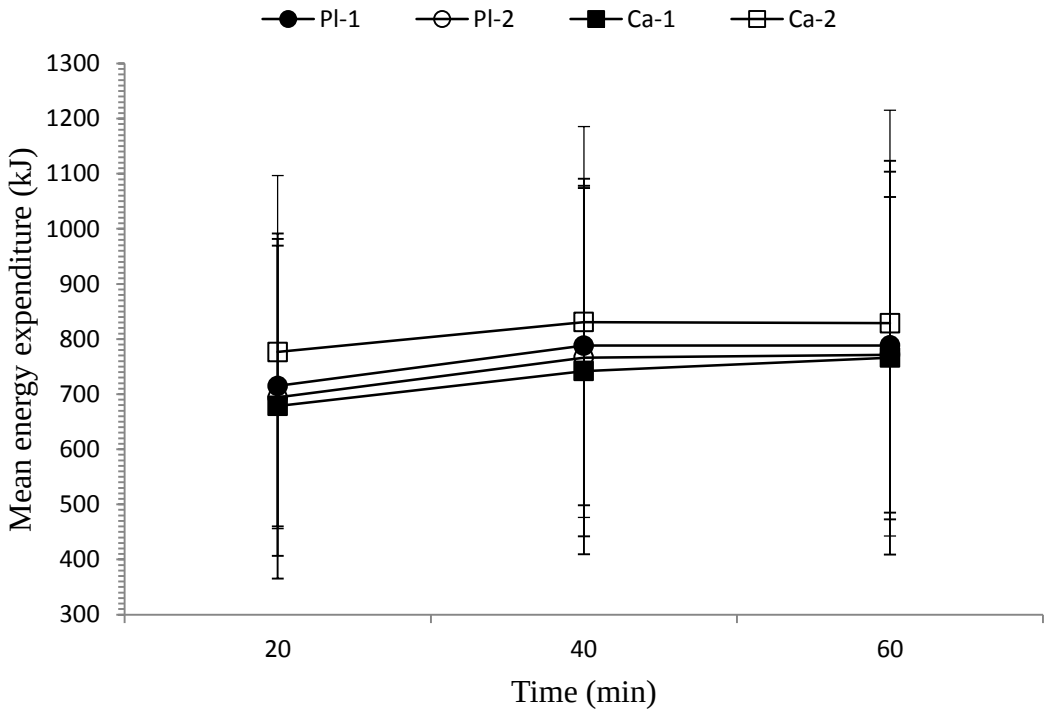


Fig. 4.7 Mean (± SD) energy expenditure during the 50%W_{peak} 60 min cycling protocol in the placebo; before (Pl-1) and after (Pl-2) and calcium; before (Ca-1) and after (Ca-2) intervention trials (n = 9)

Fig. 4.8 shows the energy expenditure in the placebo trial with values split into recreational athlete (n = 4) and well-trained athlete (n = 5) data. There was no significant change in mean energy expenditure before and after the placebo trials over the 60 min exercise period.

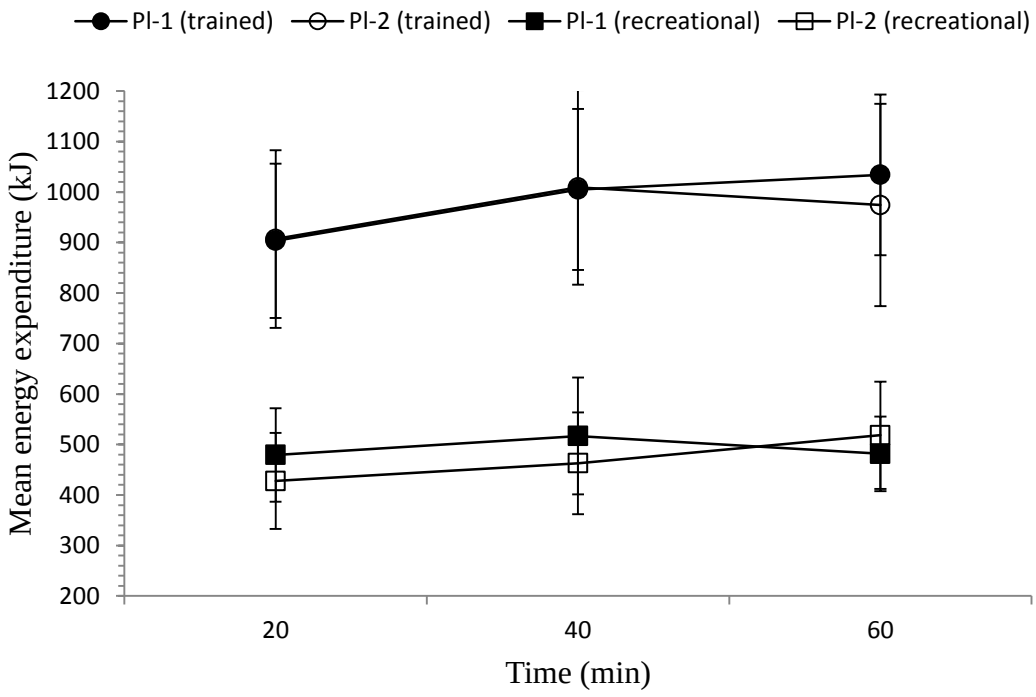


Fig. 4.8 Mean (± SD) energy expenditure during the 50%W_{peak} 60 min cycling protocol in the placebo; before (Pl-1) and after (Pl-2) trial. Split into groups of recreationally trained (n = 4) and well-trained (n = 5)

Mean energy expenditure over the exercise period of 60 min in the trial after Ca-2 was not significantly different in both recreational and the well-trained athlete (Fig. 4.9) but showed trends towards a higher energy expenditure after calcium supplementation ($p = 0.08$).

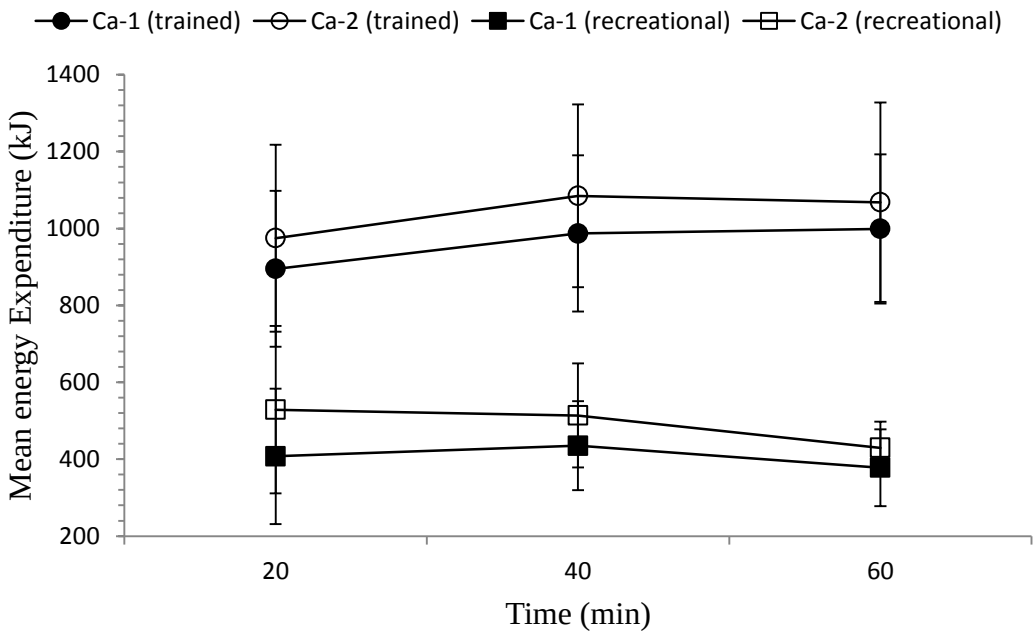


Fig. 4.9 Mean (± SD) energy expenditure during the 50%W_{peak} 60 min cycling protocol in the calcium; before (Ca-1) and after (Ca-2) intervention trial. Split into groups of recreationally trained (n = 4) and well-trained (n = 5)

4.3.5 Gas exchange

4.3.5a Oxygen uptake

There was no significant difference in the mean $\dot{V}O_2$ between all the trials (Fig.4.10). However, mean $\dot{V}O_2$ was higher by a mean of 2.98 ± 1.17 ml/kg/min (p = 0.15) in the first 40 min in Ca-2 compared to all the other trials and dropped to a mean increase of 1.37 ± 2.56 ml/kg/min (p = 0.22) in the last 20 min of the exercise There was a significant main effect of time on mean $\dot{V}O_2$ values during all exercise time points compared to resting (start) values (p = 0.01).

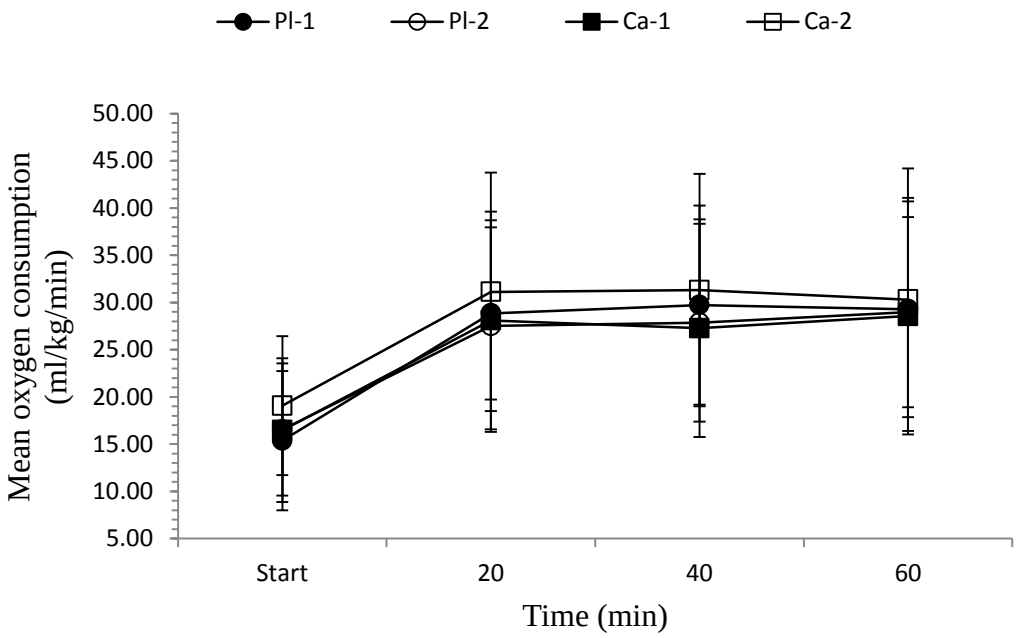


Fig. 4.10 Mean (± SD) oxygen consumption during the 50%W_{peak} 60 min cycling protocol in the placebo; before (Pl-1) and after (Pl-2) and calcium; before (Ca-1) and after (Ca-2) intervention trials (n = 9)

4.3.5b Respiratory Exchange Ratio (RER)

The RER values at the beginning and during 60 min of exercise in all the trials are presented in Fig. 4.11. Two participants displayed excessively high levels of carbon dioxide production and were thus excluded from the RER calculations. Following calcium supplementation, mean RER showed a downward trend throughout the exercise hour compared to the other trials ($p = 0.11$). However there was a significant main effect of time on RER values during exercise compared to the values at the start ($p = 0.001$). Post-Hoc analysis also revealed differences between time points 20 and 60 ($p = 0.01$) and 40 and 60 ($p = 0.01$) but not 20 and 40 ($p = 0.08$).

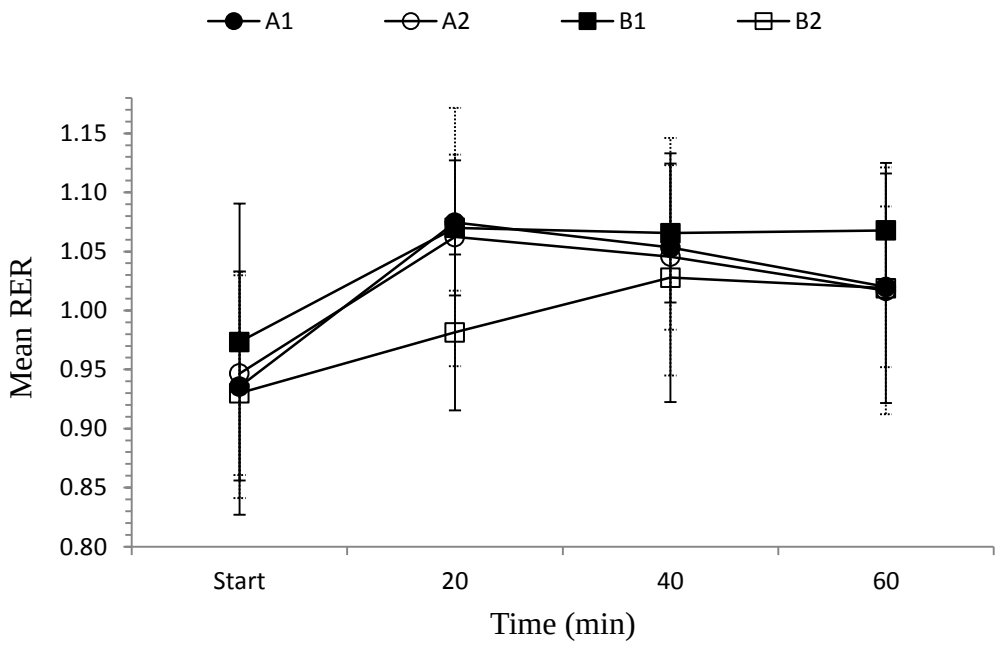


Fig. 4.11 **Mean (± SD) respiratory exchange ratio during the 50%W_{peak} 60 min cycling protocol in the placebo; before (Pl-1) and after (Pl-2) and calcium; before (Ca-1) and after (Ca-2) intervention trials (n = 9)**

Furthermore, when the RER data was split between the recreational and the well-trained athletes, the downward trend in the RER values was presented by the well-trained athletes after Ca-2 compared to all other trials (Fig. 4.12).

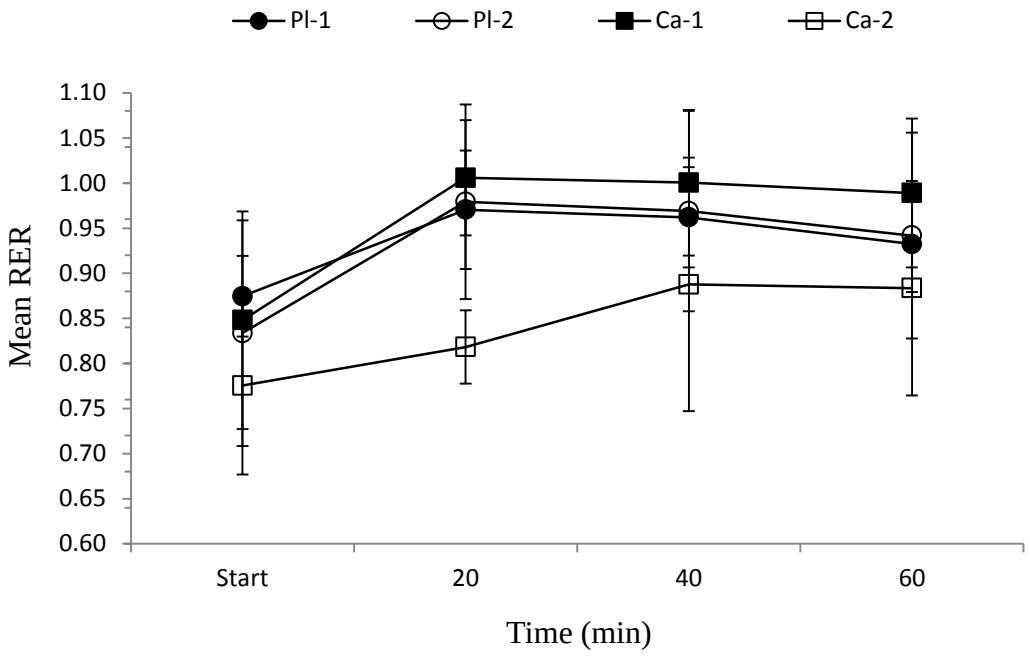


Fig. 4.12 Mean (± SD) respiratory exchange ratio during the 50%W_{peak} 60 min cycling protocol in the placebo; before (Pl-1) and after (Pl-2) and calcium; before (Ca-1) and after (Ca-2) intervention trials in the well-trained participants (n = 5)

This downward trend observed in the well-trained athletes was not displayed in the recreational participants (Fig. 4.13). Here there was no effect of supplementation on the RER values.

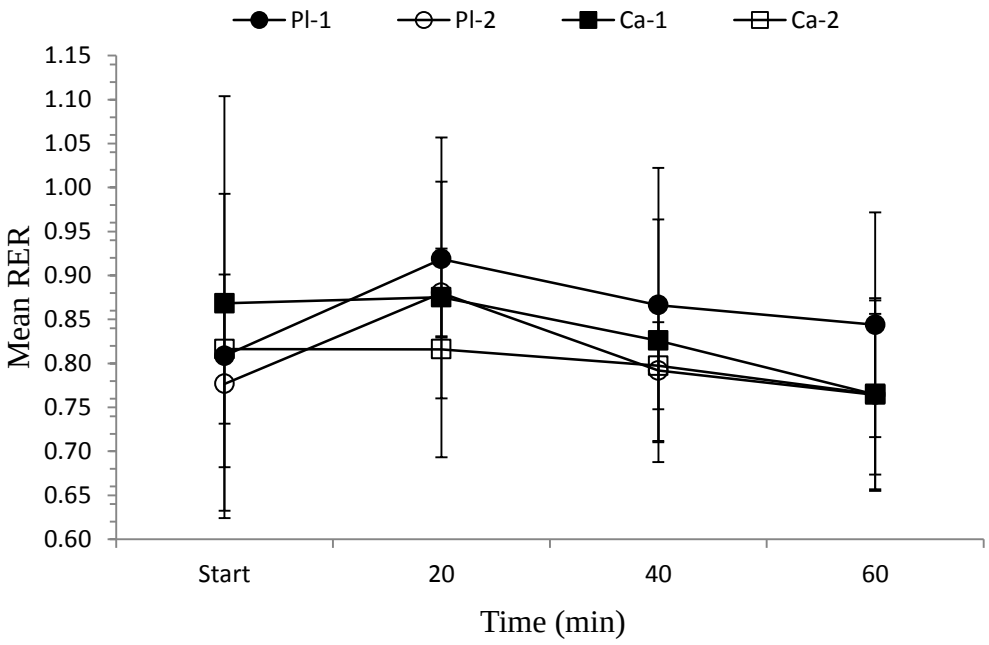


Fig. 4.13 Mean (± SD) respiratory exchange ratio during the 50%W_{peak} 60 min cycling protocol in the placebo; before (Pl-1) and after (Pl-2) and calcium; before (Ca-1) and after (Ca-2) intervention trials in recreationally trained participants (n = 4)

4.3.5c Substrate metabolism

Mean total energy expenditure (TEE) and proportion of carbohydrate and fat oxidation of all the participants are depicted in Fig. 4.14. The difference in the total energy expenditure was significantly higher in Ca-2 compared to all the other trials ($p = 0.03$). The proportion of energy expenditure via fat oxidation was larger in the Ca-2 (58.8 ± 5.9%) compared to all the other trials (Pl-1: 30.1 ± 14.5%, Pl-2: 40.1 ± 16.2%, Ca-1: 30.0 ± 12.5%); this was statistically significant ($p = 0.02$). Mean contribution of carbohydrate was significantly lower in the Ca-2 (41.1 ± 5.9%, $p = 0.03$) compared to all the other trials (Pl-1: 69.9 ± 14.5%, Pl-2: 60.0 ± 16.2%, Ca-1: 70.0 ± 12.5%).

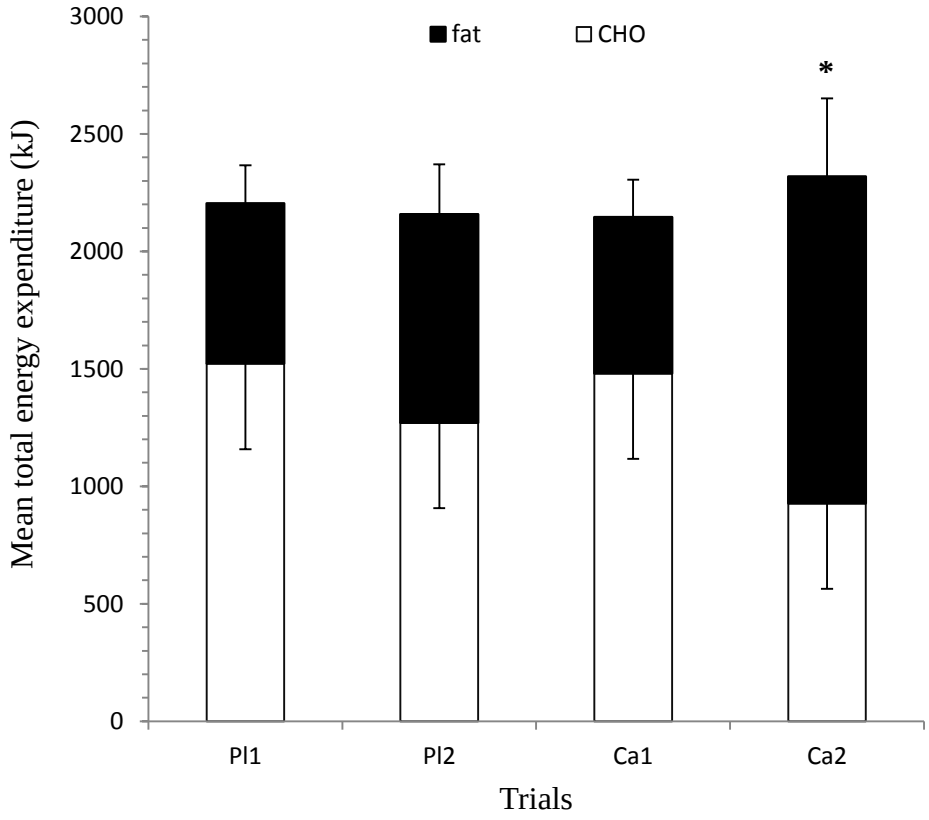


Fig. 4.14 Comparison of total mean energy expenditure and proportion of CHO (white) and fat (black) during the 50%W_{peak} 60 min cycling protocol in the placebo and calcium (n=9). *significant at $p \leq 0.05$

When the data was split into the well-trained and recreationally trained subgroups, the well-trained group displayed the same trend of increased fat oxidation rates in the Ca-2 (52.1 ± 17.9%) compared to the other trials (Pl-1: 21.9 ± 14.8%, Pl-2: 22.8 ± 22.8%, Ca-1: 26.2 ± 24.16%) (Fig. 4.15). These results did not reach statistical significance ($p = 0.08$). The remaining percentage of energy supplied by carbohydrate oxidation displayed the opposite trend of decreased CHO oxidation in Ca-2 (47.9 ± 17.9%) compared to the other trials (Pl-1: 78.1 ± 14.8%, Pl-2: 77.1 ± 22.8%, Ca-1: 86.9 ± 24.16%) ($p = 0.07$).

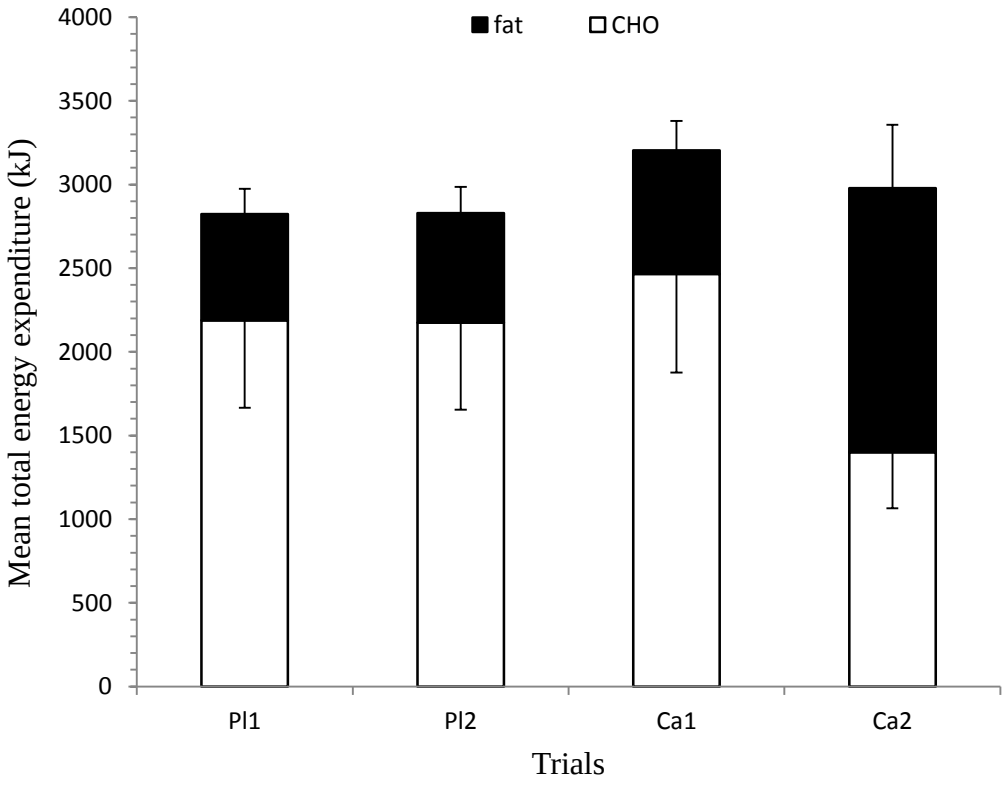


Fig. 4.15 Comparison of total mean energy expenditure and proportion of CHO (white) and fat (black) during the 50%W_{peak} 60 min cycling protocol in the placebo and calcium in well-trained participants (n=5)

There were no differences in total energy expenditure or in the proportion of CHO (Pl-1: 53.9 ± 15.8%, Pl-2: 34.0 ± 18.4%, Ca-1: 46.3 ± 18.9%, Ca-2: 33.3 ± 9.9%) ($p = 0.07$) and fat oxidation (Pl-1: 46.0 ± 15.8%, Pl-2: 66.0 ± 18.4%, Ca-1: 54.7 ± 18.9%, Ca-2: 66.7 ± 9.9%) ($p = 0.07$) rates in any of the trials with the recreationally trained subgroup (Fig. 4.16).

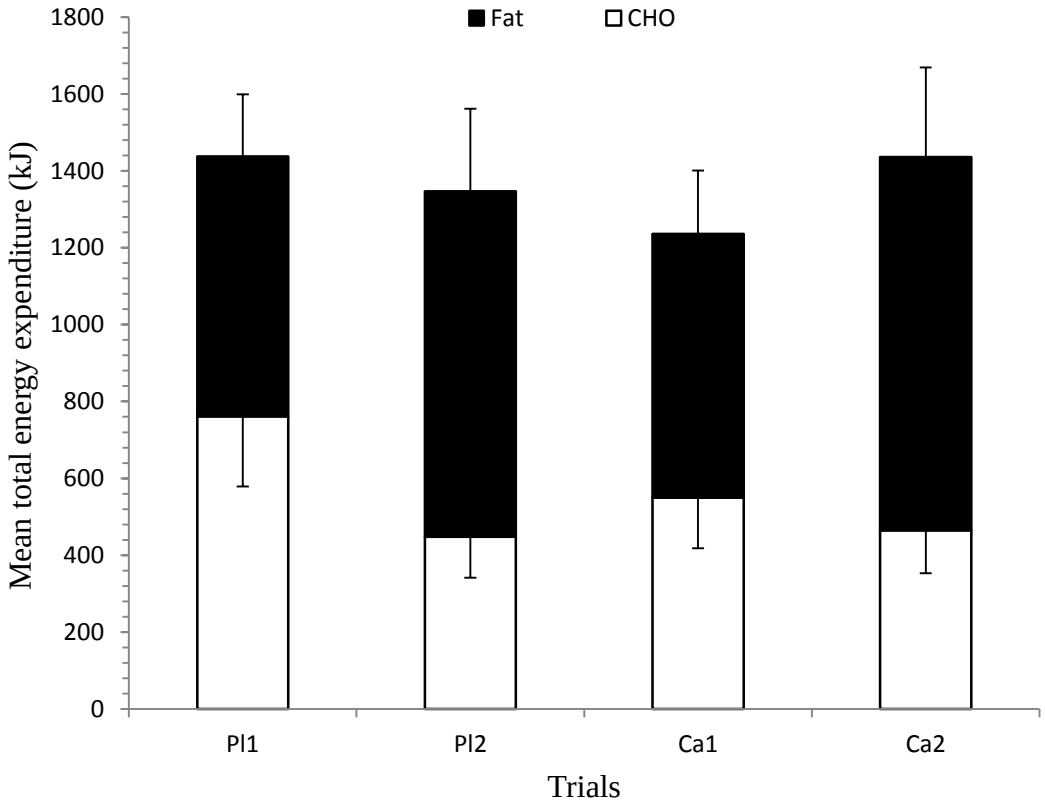


Fig. 4.16 Comparison of total mean energy expenditure and proportion of CHO (white) and fat (black) during the 50%W_{peak} 60 min cycling protocol in the placebo and calcium in recreationally trained participants (n=4)

4.3.5d Heart Rate (HR)

Mean HR at start of the exercise was lower in Ca-2 by 27 ± 11 bpm (p = 0.09) compared to the mean HR at the start of all the other trials (Fig. 4.17). Mean exercise HR was higher by 10 ± 2 bpm (p = 0.13) in Ca-2 as compared to all the other trials. However, resting HR in all trials was significantly (p = 0.001) different from their corresponding exercise HR.

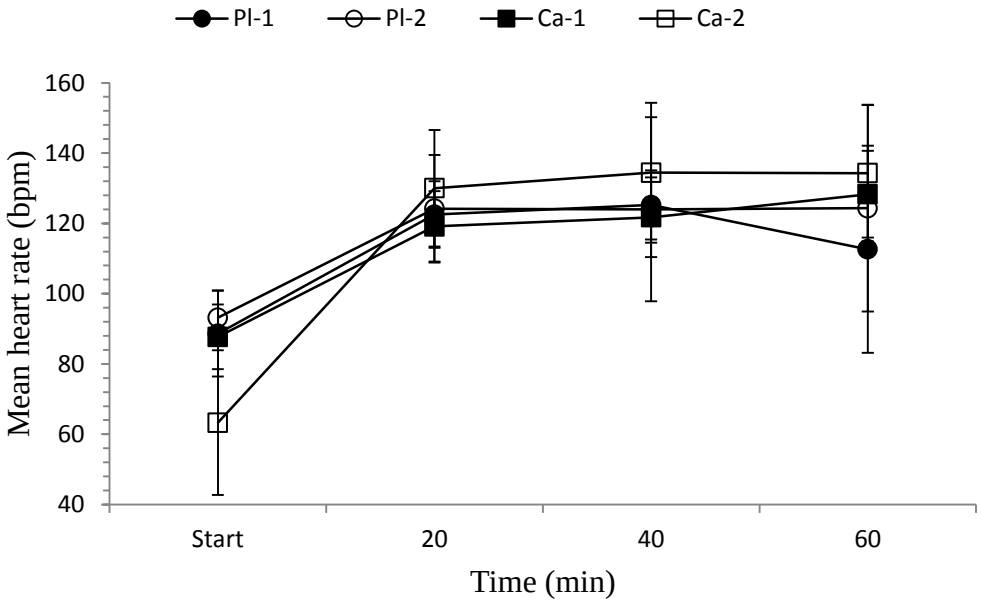


Fig. 4.17 Mean (± SD) heart rate during the 50%W_{peak} 60 min cycling protocol in the placebo; before (Pl-1) and after (Pl-2) and calcium; before (Ca-1) and after (Ca-2) intervention trials (n = 9)

4.3.6 Haematological data

4.3.6a Plasma iPTH

There was a non-significant decrease in mean resting iPTH levels after Ca-2 compared to the placebo trials ($p = 0.08$). The mean (±SD) values from all the trials are displayed in table 4.14.

Table 4.14 Mean (± SD) resting iPTH at baseline and end of each intervention in study 1.

	Pl-1 (pmol/l)	Pl-2 (pmol/l)	Ca-1 (pmol/l)	Ca-2 (pmol/l)
Mean ± SD	2.51 ± 1.16	2.77 ± 1.18	2.59 ± 0.75	2.24 ± 1.11

4.3.6b Total plasma calcium

Mean total plasma calcium is presented in Fig. 4.18. Resting plasma calcium was lower at the beginning of Ca-1 (1.97 ± 0.32 mmol/l) and Ca-2 (1.95 ± 0.32 mmol/l) as compared to the placebo trials (Pl-1 = 2.56 ± 0.27 mmol/l, Pl-2 = 2.54 ± 0.39 mmol/l; p = 0.10). There were no significant changes in total plasma calcium values among all trials and across all time points (p = 0.33).

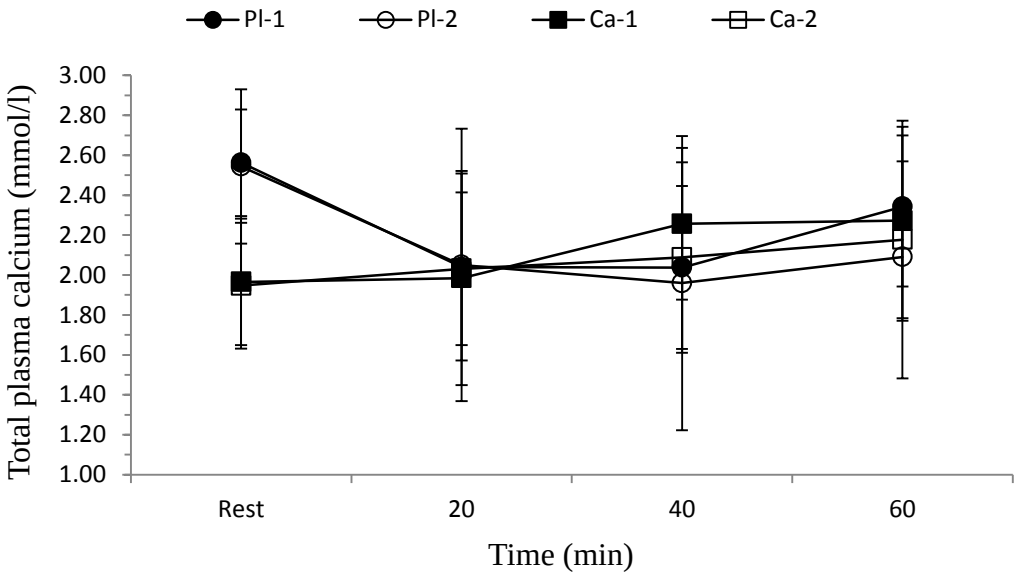


Fig. 4.18 Mean (± SD) total plasma calcium during the 50%W_{peak} 60 min cycling protocol in the placebo; before (Pl-1) and after (Pl-2) and calcium; before (Ca-1) and after (Ca-2) intervention trials (n = 9)

4.3.6c Plasma non-esterified fatty acids (NEFA)

There was a significant main effect of time in the mean plasma NEFA values at rest from those that of exercise in all trials (p = 0.02) (Fig. 4.19). Even though mean plasma NEFA in Ca-2 was higher compared to all the other trials throughout the 60 min exercise (p = 0.07) this did not reach statistical significance.

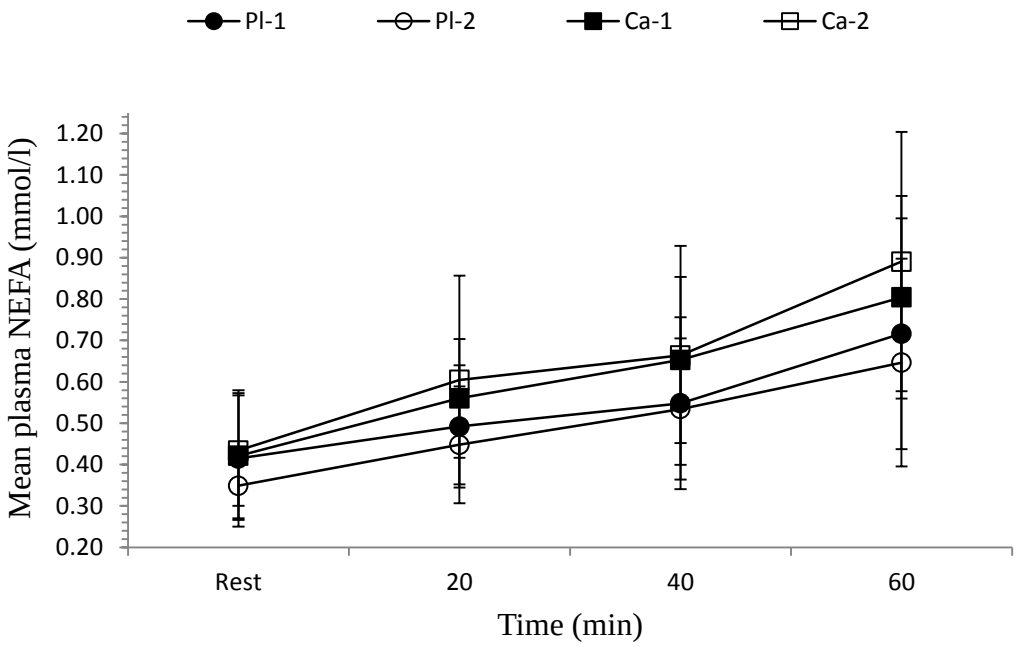


Fig. 4.19 Mean (± SD) plasma non-esterified NEFAs during the 50%W_{peak} 60 min cycling protocol in the placebo; before (Pl-1) and after (Pl-2) and calcium; before (Ca-1) and after (Ca-2) intervention trials (n = 9)

4.3.6d Plasma triacylglycerols (TAG)

Mean resting TAG values was significantly lower than exercise TAG values in all trials (main effect of time, p = 0.001) (Fig. 4.20). There was no significant difference in the plasma TAG values during exercise between all trials.

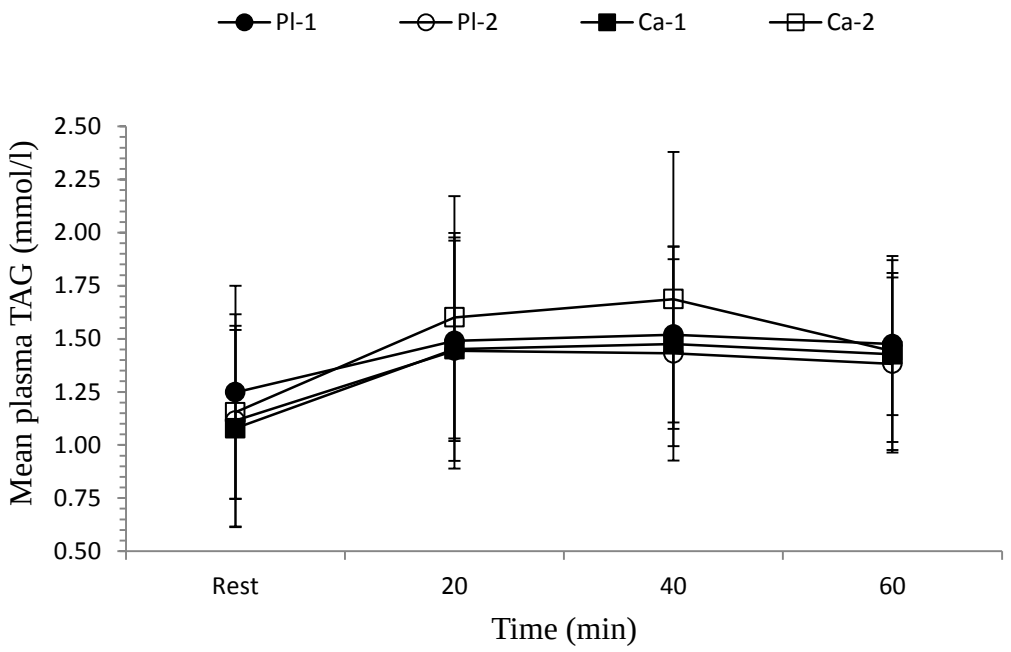


Fig. 4.20 Mean (± SD) plasma triacylglycerides during the 50%W_{peak} 60 min cycling protocol in the placebo; before (Pl-1) and after (Pl-2) and calcium; before (Ca-1) and after (Ca-2) intervention trials (n = 9)

4.3.6e Plasma glucose

Mean plasma glucose values did not show much variation between the trials and remained tightly regulated between 4.0 – 5.0 mmol/l (p = 0.44) (Fig. 4.21). Also mean plasma glucose did not alter from rest to exercise (p = 0.22, main effect of time).

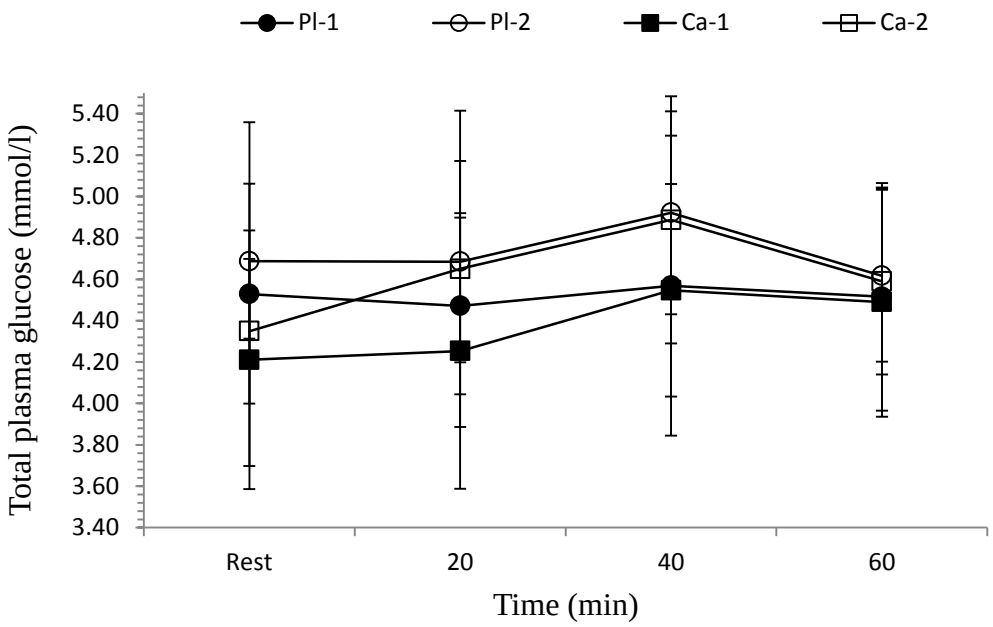


Fig. 4.21 Mean (± SD) plasma glucose during the 50%W_{peak} 60 min cycling protocol in the placebo; before (Pl-1) and after (Pl-2) and calcium; before (Ca-1) and after (Ca-2) intervention trials (n = 9).

4.3.6f Plasma lactate

Mean resting plasma lactate values in all trials are presented in Fig. 4.22. These were significantly lower than their corresponding exercise values ($p = 0.01$, main effect of time). There were no significant differences between lactate values of all trials ($p = 0.08$, main effect of supplementation). However, plasma lactate values during Ca-2 were higher than all the other trials and increased in the first 20 min of exercise and did not decrease for the entire hour. All the other trials, which saw a decrease in lactate values at 40 min time point after the initial increase at 20 min, before stabilising for the rest of the hour.

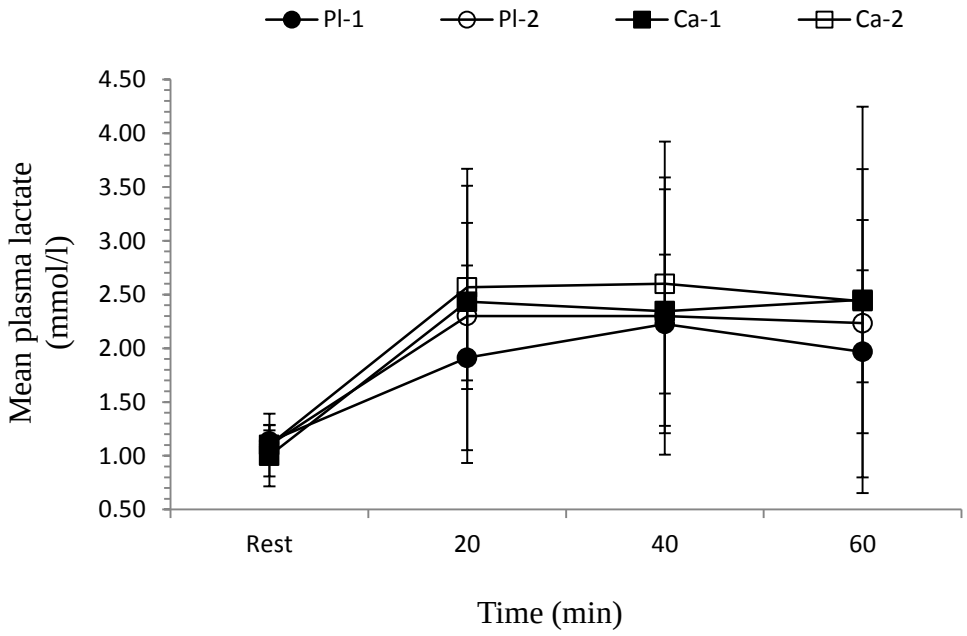


Fig. 4.22 Mean (± SD) plasma lactate during the 50%W_{peak} 60 min cycling protocol in the placebo; before (Pl-1) and after (Pl-2) and calcium; before (Ca-1) and after (Ca-2) intervention trials (n = 9)

4.3.7 Rating of Perceived Exertion (RPE)

There were no significant differences in RPE rating between any of the trials (Fig.4.23). Resting RPE was significantly different from all exercise values ($p = 0.001$, main effect of time), but there were no significant differences across all time points of exercise in RPE rating in any trials ($p = 0.43$).

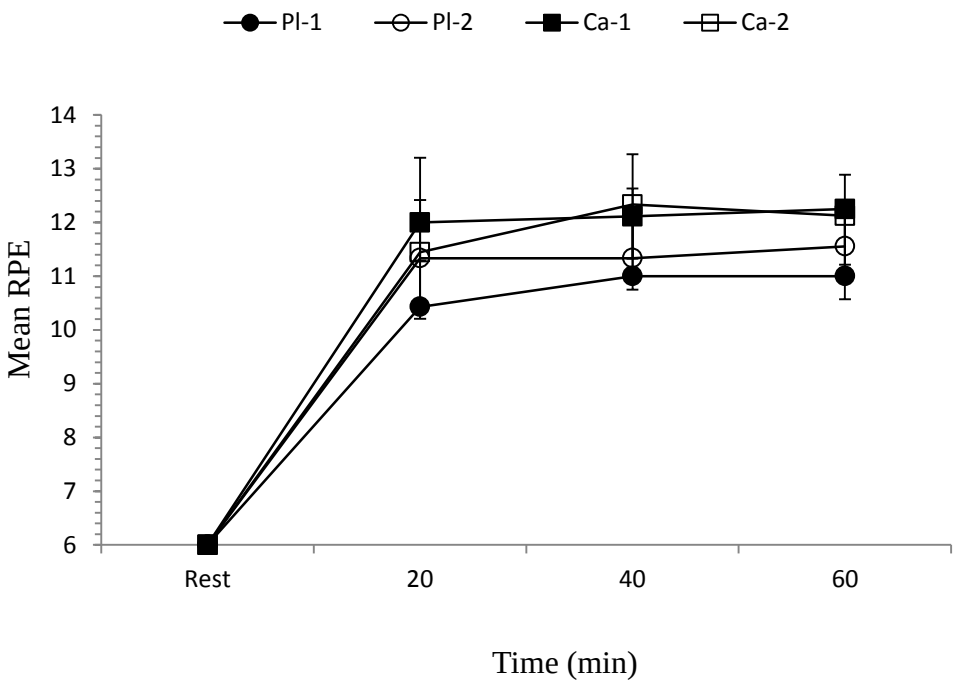


Fig. 4.23 Mean (± SD) RPE during the 50%W_{peak} 60 min cycling protocol in the placebo; before (Pl-1) and after (Pl-2) and calcium; before (Ca-1) and after (Ca-2) intervention trials (n = 9)

4.4 Discussion

The principle aim of this study was to determine the effects of calcium supplementation for four weeks on substrate metabolism during submaximal exercise and to determine body composition changes. The main findings suggest a improved but non-significant shift in substrate metabolism towards a greater fat oxidation rate during the 60 min cycling trial after calcium supplementation compared to the placebo trials. This trend was amplified in well-trained athletes and not the recreationally trained athletes. In addition to changes in substrate oxidation rates, there was a significant reduction in body fat percentage of the participants with a concurrent increase in lean mass during the calcium supplementation trial. On further analysis this trend of body composition change in the calcium supplementation trial was attributed to the changes in the well-trained athletes' body composition only.

4.4.1 Fat metabolism

Findings from this study showed a decrease in RER values during the entire exercise hour at the end of Ca-2 compared to the placebo trials. This suggests that there was a shift towards oxidative metabolism following calcium supplementation. Oxygen consumption data supports this, with $\dot{V}O_2$ values being higher in the trial at the end of calcium supplementation period than before. Comparing the gas analysis results with blood indices of metabolism there seems to be some commonality in the inference of higher levels of appearance of oxidative substrates in plasma such as NEFA and TAG during Ca-2. Men'shikov (2004) reports a strong correlation (r = 0.72, $p < 0.05$) between plasma NEFAs, changes in blood calcium and oxygen

consumption during exercise in well-trained athletes. This study did not make any dietary or supplementary intervention and recorded the acute responses of the blood and gas parameters during exercise of low to moderate intensity for 90 min. The athletes with low plasma calcium displayed low plasma NEFA and a high oxygen consumption value. Moreover, the non-athletes did not display such correlations. NEFA following calcium supplementation continued to increase during the entire exercise hour as one might expect from a moderate intensity protocol. These findings are in agreement with Wolfe et al. (1990) where they showed that the rate of appearance of NEFA rises steadily throughout moderate intensity exercise (40%$\dot{V}O_{2max}$, for 2 hours). Even as the NEFA levels in all trials rose throughout the exercise hour, the increase was highest during the entire exercise period in trial Ca-2. This is a further indication of continued higher rates of lipolysis following calcium supplementation throughout the exercise period.

Rate of appearance of NEFA in the blood can only be used as a surrogate measure to understand lipolytic activity. Previous studies by Hodgetts et al. (1991) found that the ratio of NEFAs to albumin in venous blood of athletes coming from subcutaneous adipose tissue increased from 2:1 at rest to nearly 6:1 at the end of exercise. Klein et al. (1994) also observed that during the first 20 min of exercise at 45%$\dot{V}O_{2max}$, lipolytic rate is approximately twice the rate of NEFA oxidation. This is particularly the case in this study as well since the highest increase in a NEFA and TAG is seen in the first 20 min of exercise and the post calcium supplementation trial shows the largest increase in these values, especially as the resting NEFA value in the trial following calcium supplementation was lower than the other trials. This could be the effect of increased dietary calcium that may have magnified the lipolytic rate in this trial. Various studies looking at the relation between the rate of

lipolysis and the rate of fat oxidation have suggested that at low to moderate intensity the relationship between lipolysis and fat oxidation is more or less linear as delivery of NEFA to the mitochondria is not obstructed either by restricted blood flow (Friedlander et al., 1999, Watt et al., 2002) or by albumin binding capacity (Romijn et al., 1993, Klein et al., 1994, Watt et al., 2002). Therefore increased availability of NEFA at the start of the exercise could lead to interesting avenues of supporting the hypothesis of calcium in glycogen sparing action during low to moderate intensity exercise of long duration. This can be an avenue worth exploring from the perspective of developing appropriate training strategies to maximise fat oxidation and glycogen sparing during events where glycogen availability may become a limiting factor in performance.

Increased TAG appearance in the blood could also suggest towards the increased demand for NEFAs. The results from this study show the increase in TAG in plasma during Ca-2 trial in the first 20 min of exercise is the highest among all the trials. This could be a strong indication of increased mobilisation of fatty substrates at the onset of exercise. Few studies have evaluated the contribution of plasma triacylglycerols to total energy production. The available data suggests that during the resting conditions, plasma triacylglycerols may account for 5-10% of the total fat oxidation (Wolfe et al., 1985, Ryan et al., 1965, Klein et al., 1994). There is also indirect evidence that only a small fraction of total energy produced is derived from plasma triacylglycerols during exercise (Turcotte et al., 1992). Even though direct measurement of TAG oxidation during exercise has not been reported in the literature in this study plasma TAG increased during the exercise hour; not in a dissimilar pattern to NEFA increases. However in all the trials TAG seemed to decrease in the last 20 min of exercise. This decrease was higher during Ca-2 as

compared to Ca-1. Further investigation is required if this effect of decreased plasma TAG levels is related to abundant supply of NEFA towards the end of the exercise hour and if that helps in understanding the effect of lipid substrates in the blood during exercise.

4.4.2 Glucose metabolism

Plasma glucose remained tightly regulated between 4.0 – 5.0 mmol/l during all the trials. This is in conjunction with trials conducted with a similar fed state (moderate carbohydrate meal 60-90 min) prior to exercise at 50%$\dot{V}O_{2max}$ (Coyle, 1995b, Romijn et al., 1993). One of the reasons proposed for this is the neuroendocrine control of both glucose production by the liver and glucose uptake by the muscle and muscle glycogenolysis (Wasserman, 1995). However, as dietary calcium is postulated in the interaction of insulin and catecholamines (Underhill, 1916) it was expected that there would be some metabolic consequences as a result of increased dietary calcium. When calcium levels in the plasma are stable blood glucose remains stable; whereas with changes in plasma calcium levels these regulations are affected (Underhill, 1916). It is possible that at a moderate intensity (50%$\dot{V}O_{2max}$) where the role of glucose-fatty acid cycle is well regulated by the availability and requirement of substrates, the mechanism that may involve the calcium does not play a mitigating role for glucose appearance and this utilization. It has been suggested (Hoelzer et al., 1986) that there is a redundancy in the control systems that regulate glucose fluxes because deficiency of one system need not necessarily lead to hypoglycaemia.

4.4.3 Energy expenditure

In the trial following calcium supplementation the participants expended significantly more energy than all the other trials. This supports the metabolic inefficiency model suggested by Zemel (2002). Core body temperature was not measured in this study. This could have perhaps given some more information regarding the nature of calcium action in increasing metabolic inefficiency and in some way help comment on the suggested role of $1,25(OH)_2D_3$ and its dose responsive inhibitory effect on UCP 2 expression and in effect core body temperature. However, human studies have not demonstrated this metabolic inefficiency proposed by *in-vitro* studies (Jacobsen et al., 2005, Boon et al., 2005). Kozyreva et al. (1983) has reported an adaptation to low temperatures is associated with decrease in blood calcium and an increased sensitivity of β-adrenoreceptors to norepinephrine. Since total energy expenditure is calculated as a function of oxygen consumption (Whaley et al., 2006), the energy expenditure during exercise could have increased as a result of change in substrate utilization with a shift towards higher fat oxidation rates in the trial following four weeks of calcium supplementation. Most studies looking at the role of calcium in energy expenditure have not applied an exercise protocol. It is therefore difficult to conclusively say if the energy expenditure increase after calcium supplementation was due to changes in substrate metabolism or via another mechanism/s involving resting metabolic rate changes such as the role of UCP in adipocytes and skeletal muscle.

4.4.4 iPTH and calcium metabolism

In response to the dietary calcium supplementation resting iPTH levels decreased in the Ca-2 trial, but this did not reach statistical significance. Studies looking at bone markers in athletic populations have reported similar decreases in iPTH levels with calcium supplementation (Riggs et al., 1998) and/or calcium and vitamin D supplementation (Dawson-Hughes et al., 1997, Pfeifer et al., 2001). Guillemant et al. (2004) have also demonstrated a single load of calcium (1g) suppressed osteoclastic activity acutely induced by an endurance cycling exercise. This is perhaps the consequence of decreased iPTH levels due to calcium intake.

However, a physiological response to moderate exercise on plasma iPTH levels is associated with a increased plasma values (Ljunghall et al., 1986a) without calcium supplementation. Ljunghall et al. (1988) have reported ~25% increases in plasma iPTH levels of the same magnitude as that of a hypocalcemic test, immediately after exercise. Thorsen et al. (1997) have reported an increase in plasma iPTH levels with concurrent decreases in plasma ionised calcium levels at 1 hour ($p < 0.001$) and 72 hours ($p < 0.05$) and a significant increase of iPTH at 24 hour ($p < 0.01$) and 72 hours ($p < 0.05$) after the test. In other short term studies by the same group of researchers, where exercise was performed with varying intensities for periods between 2 min and 1 hour, normal participants did not display any consistent changes in plasma iPTH (Ljunghall et al., 1985, Ljunghall et al., 1984). However, during a 5 hour bicycle exercise study there was a small decrease in iPTH (Ljunghall et al., 1986b). Thus it appears that the normal response to exercise on iPTH is dependent on the duration of exercise. The intensity of exercise influences calcium levels and thus iPTH levels due to increased acidosis (Ljunghall et al., 1985). One of the possible explanations for decreased plasma ionised calcium levels during

exercise, that stimulates increases in iPTH levels could be due to increases in complex binding of free calcium ions as well as enhanced binding of calcium to albumin molecules with the increased plasma NEFA during prolonged exercise (Zaloga et al., 1987). The stimulation of cAMP and catecholamine levels due to exercise has also been implicated in the changes of plasma ionised calcium levels during exercise due to their effect on increased plasma NEFA during exercise. Post exercise iPTH levels were not measured in this study. However, it is possible that those may remain unchanged or even display the trend of reduction seen at resting levels in the trial following calcium supplementation due to the longitudinal nature of this study.

4.4.5 Changes in body mass and composition

There was a decrease in body mass and fatness over the four week intervention period following calcium supplementation. Recent findings from other studies support these findings. In a two year intervention study by Lin et al. (2000) 54 normal weight women participating in an exercise intervention, the dietary calcium to energy intake ratio was a significant predictor of changes in both body weight and body fat. In one large trial of the effect of calcium supplementation on bone, in which elderly women were randomly assigned to either placebo or 1.2 g/d of calcium carbonate, Davies et al. (2000) analysed this data retrospectively for changes in body weight and found that the calcium supplemented group lost 0.671 ± 0.112 kg/year as compared to the placebo group who lost 0.325 ± 0.110 kg/year, reporting an estimating calcium treatment difference of 0.346 kg/year ($p < 0.05$). The overall relationship derived from this reanalysis indicates a calcium intake increase of 1 g/d

is associated with an 8 kg reduction in body weight (Davies et al., 2000). The body mass reduction seen in this study is substantially higher as compared to the meta-analysis of 780 women from five separate clinical trials by Davies et al. (2000). One possible explanation for this could be that the proposed mechanism for the effect of calcium intake on changes in weight and fat mass would support that exercisers would have increased lipolysis during training and subsequent physiological adaptations; thus, the effect may be greater in athletes.

The BMI of the participants reduced by 0.19 ± 0.26, which was not a significant decrease but not much emphasis, will be placed on BMI data. This data should be viewed in light with the body composition data. The lower fat mass generally biases the value towards overweight or even obese when the participants in this study in reality weigh more due to a higher lean tissue mass in athletes as compared to sedentary individuals. This is more important as changes in body composition were particularly pronounced in the well-trained athletes. One possible reason for this distinction could be due to the fact that athletes due to increased expenditure and better absorption rates (Zittermann et al., 2000) and traditionally higher calcium loss through increased sweat rates (Klesges et al., 1996, Leiper et al., 1996) may have benefitted from the calcium supplementation more than the recreationally trained athletes.

The case for improved body composition upon calcium supplementation of people on a habitually low calcium intake has been made repeatedly in the literature (Davies et al., 2000, Heaney et al., 2002, Teegarden, 2003). No significant differences between habitual calcium intakes were found in both the groups in the study. The participants' habitual calcium intake was proportional to the mean total energy intake in both the sets of diet diaries (r = 0.65). However, other aspects of calcium

metabolism such as sweat losses and energy balance as a result of higher training volumes (and thus increased fat oxidation) may have also played a part in the change in body composition of the well-trained athletes over the recreationally trained. An interesting caveat on the differences between the well-trained and the recreationally trained athlete groups in this study was that due to the criteria for sub grouping ($\dot{V}O_{2max}$ > 50 ml/kg/min) there was an unintentional split in gender within the same sub grouping (recreationally trained = female, well-trained = male). It has been shown in a few studies that males have responded better than females to the increased dietary calcium intervention with relation to body mass (Vergnaud et al., 2008, Heaney et al., 2002) and body composition (Vergnaud et al., 2008, Loos et al., 2004) better than women. Therefore, another possible explanation for the differences in the two sub groups in the study may be that the intervention had a larger effect on males compared to that in females.

It is important to mention that studies that have looked at dairy calcium intake have reported much larger changes in body mass and composition (Azadbakht et al., 2005; Barr, 2003; Davies et al., 2000; Harvey-Berino et al., 2005; Lin et al., 2000; Lorenzen et al., 2007; Pereira et al., 2002; Teegarden et al., 2003; Zemel et al., 2005; Zemel et al., 2003). The Coronary artery risk development in young adults (CARDIA) study evaluated the associations between dairy product intake and the incidence of the major components of the insulin resistance syndrome, including obesity, in a 10 year study of 3157 black and white adults. The cumulative incidence of obesity in those who started the study in the overweight category was significantly reduced from 64.8% in those who consumed the least amount of dairy foods to 45.1% in those who consumed the highest amount of dairy foods (Davies et al., 2000). Pereira et al. (2002) explained these differences solely by dairy intake and not

altered by adjustment for dietary calcium, which probably explains the other beneficial substances in dairy products mentioned previously that may explain the increased weight loss in those studies.

4.4.6 Conclusions

The main finding from this investigation was that total energy expenditure was significantly higher in the trial following calcium supplementation. There was no statistically significant difference in the metabolites in the blood or fat oxidation during exercise among all the trials (thus refuting hypothesis i). Even though most of the results did not show statistical significance the trend clearly warrants more investigation with well-trained athletes since the changes seem more pronounced in well-trained athletes compared to recreational participants.

Also, this study being longitudinal in nature allowed for changes in body mass and composition in the participants that seem to clearly support most of the data in the literature on calcium and its anti-obesity effects. There was a statistically significant difference in decrease in total body mass and fat mass of the participants after calcium supplementation (thus supporting hypothesis ii). Once again these changes in body composition were markedly pronounced in well-trained athletes compared to the recreational participants.

Put together these findings present a strong case for further investigation into the *in-vivo* effects of calcium in fat metabolism, its effects on body mass and composition, from a view to improve performance. Thus future studies aimed at

highly trained participants involving performance tests, may highlight the direct ergogenic effects of supplementary calcium intake.

CHAPTER 5

EFFECTS OF CALCIUM SUPPLEMENTATION ON PERFORMANCE OF A TWENTY FIVE MILE CYCLING TIME TRIAL

5.1 Introduction

Increased dietary calcium has been shown to have positive correlation with fat metabolism (Xue et al., 2001) and subsequently decreased body fat mass (Zemel, 2003, Zemel et al., 2000, Gunther et al., 2006). It was demonstrated in the previous study that sub maximal exercise at a set intensity (50%W_{peak}) increased total energy expenditure after four weeks of calcium supplementation compared to the placebo trial. Also, indices of fat metabolism indicated a positive effect of calcium supplementation on fat oxidation during a steady state submaximal exercise session lasting for 60 minutes. In addition to changes in substrate metabolism, the body composition of the participants in the previous study improved towards an increase in lean mass and a decrease in fat mass during the four weeks of supplementation. These changes were more pronounced in the well-trained athletes compared to the recreational participants. Thus the current study was designed to further establish the relevance of these shifts in substrate metabolism from the perspective of improvement in real time performance. The results obtained from the previous study

led to an extension of the premise of ergogenicity of calcium and the need to study the external validity of those results derived from controlled sub maximal tests. It has been argued by Atkinson and Nevill (2001) that improvements in performance based on relevant physiological parameters might be smaller than those exhibited by the physiological variables. Since calcium regulation is very tightly controlled in all the organs including blood, it would be interesting to see the effects of increase in calcium intake on direct performance related variables such as power output and time-to-completion via the use of time trial tests.

Thus, the aim of this experimental trial was to study the effects of four weeks of calcium supplementation specifically on performance during a long duration endurance event (twenty five mile cycling time trial (25 TT)). The secondary aim was to study its effect on body composition changes during the four weeks of supplementation on highly trained athletes.

5.1.1 Hypotheses

iii. It is hypothesised that calcium supplementation of 1000 mg/day for four weeks improves performance in a 25 mile time trial.

iv. It is also hypothesised that calcium supplementation of 1000 mg/day for four weeks reduces body fat mass of highly trained cyclists.

5.2 Methods

5.2.1 Participants

Ten male healthy well-trained cyclists (time trialists) were recruited from local cycling clubs. Inclusion criteria as described in section 3.1 were met. An exclusion criterion was set at 10 mile time-trial time-to-finish of ≥ 25 min in the past season. The mean (± SD) physical characteristics of the participants are given in Table 5.1.

Table 5.1 Mean (± SD) physical characteristics of participants in study 2

	Age (years)	Weight (Kg)	Height (cm)	$\dot{V}O_{2peak}$ (l/min)	$\dot{V}O_{2peak}$ (ml/kg/min)	$W_{peak(aerobic)}$ (W)	PO_{LT} (W)
Mean (± SD)	35.8 ± 11.3	73.80 ± 9.31	176.0 ± 7.10	4.59 ± 0.85	62.70 ± 6.43	350 ± 42	238 ±16

5.2.2 Experimental design

The study was designed as a randomised, single blind, test - retest intervention. Participants were initially assigned to a four week calcium intervention (1000 mg elemental calcium (citrate)/day. The following year, four out of the ten participants returned to perform the study without any intervention (nocebo). The study was designed as a single blind intervention to ensure the validity of the data was not compromised due to the long waiting period between data collection. Participants

were not informed of the single blind nature of the study and were led to believe that the intervention was double blind and they were randomly assigned to either a calcium or placebo group. The study was repeated at the same time of the year (October to January) to coincide with the end of the racing calendar and therefore matched training regimes. This allowed the changes observed in the intervention not to be caused primarily due to increase in training intensity or load that occurs during the racing season. In addition the October to January period is generally used as a low training period by most competitive cyclists (Sassi et al., 2008, White et al., 1982a). Therefore any improvements observed could be attributed to a large extent on the intervention.

As with the earlier intervention the capsule formulation procedure was adhered to as described in section 3.2.8.

5.2.3 Testing protocols

5.2.3a $W_{peak\ (aerobic)}$ test

All participants underwent a $W_{peak\ (aerobic)}$ test one at a time in order to compare their time trial performance power output with their peak power output. Participants reported to the laboratory in a post prandial state of at least one meal consumed about 60 to 90 min prior to testing. After setting up of cycle ergometer and emptying of bladder, the test was commenced and conducted as described in section 3.3.4.

5.2.3b Time trials

Participants performed two 25 TT, one at baseline before calcium supplementation (25TTC1) and one at the end of the four week intervention period (25TTC2). On each occasion the participants arrived post prandial after consumption of the same meal at least 60-90 min prior to testing. Upon arrival they underwent the anthropometry measurements as described in section 3.2.5a and 3.2.5b and resting blood measurements as described in section 3.2.4b for analysis of lactate, plasma glucose and NEFA. The tests were performed as described in described in section 3.3.5. Water was allowed *ad-libitum* throughout the tests. No verbal or other forms of encouragement were given during the tests.

At the same time in the following year participants (n = 4) repeated the same study with a nocebo intervention. They performed a baseline test (25TTP1) and repeated the time trial after four weeks (25TTP2).

5.2.3c Dietary and physical activity records

All participants were asked to keep a seven day diet diary and physical activity log as described in section 3.2.6 during any typical week of the intervention and were asked to repeat the same in the following year during the nocebo intervention.

A schematic representation of the study design is presented in Fig. 5.1.

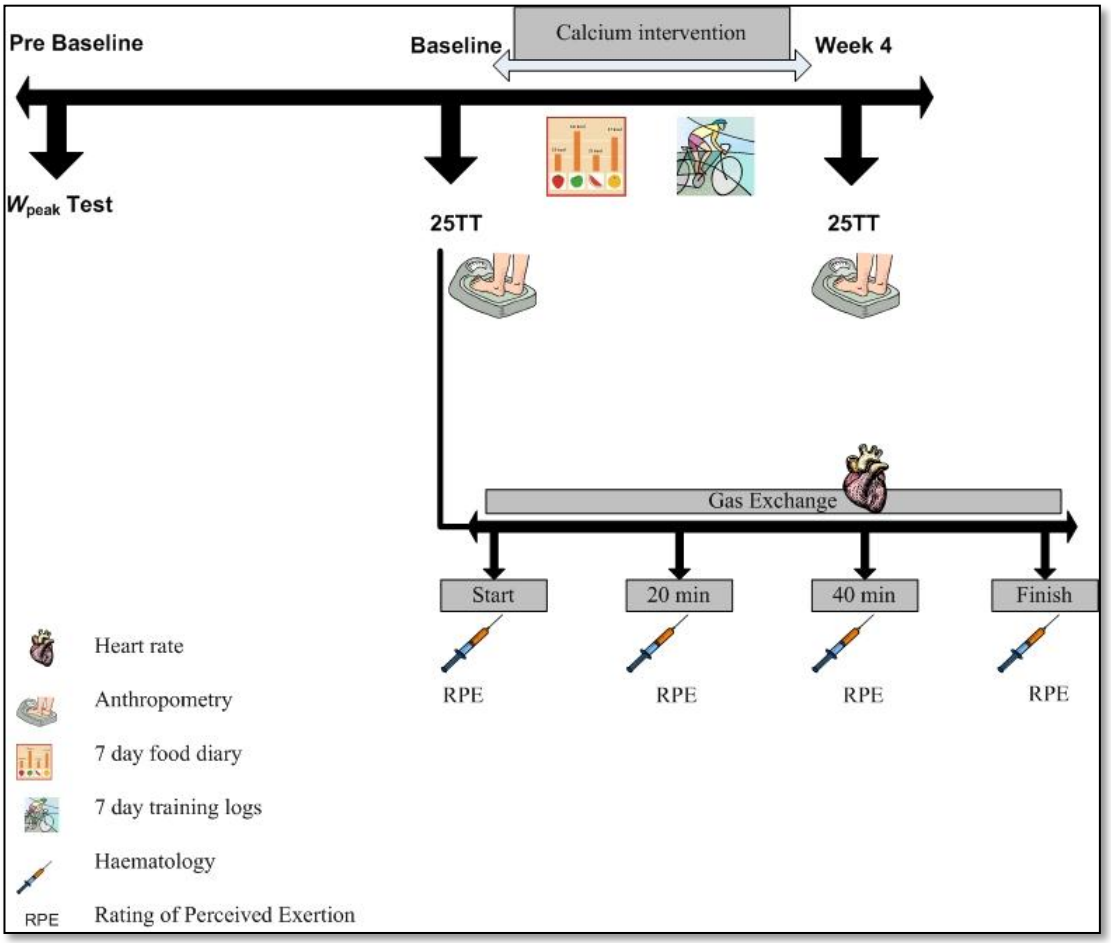


Fig. 5.1 Schematic representation of experimental protocol within each intervention

5.2.3d Statistical analysis

All data was analysed and statistical analysis conducted as described in section 3.5. Mean differences were analysed for intra-trial changes and not inter-trial changes. This was done to measure the changes due to calcium supplementation from baseline and after four weeks.

5.3 Results

5.3.1 Anthropometry

Mean percentage change of all anthropometric values from the calcium intervention are presented in table 5.2. Following calcium supplementation for four weeks mean total body mass of the participants increased by 0.22% (p = 0.12), However, body composition improved favourably by a decrease in fat mass (-0.81%, p = 0.07) with a concurrent increase in lean mass (0.81%, p = 0.07). These changes in body composition did not reach statistical significance.

Table 5.2 Mean (±SD) anthropometric changes in participants of study 3 following calcium supplementation for four weeks

n = 10	25TT C1 (mean ±SD)	25TT C2 (mean ± SD)	Difference; ± 90%CI	*d*
Total Mass (kg)	73.78 ± 9.29	73.94 ± 8.70	-0.16; ± 0.31	0.02
BMI (kg/m^2)	23.78 ± 2.25	23.84 ± 2.12	-0.06; ± 0.07	0.01
Fat Mass (kg)	11.08 ± 4.64	10.50 ± 4.86	-0.58; ± -0.11	-0.12
Lean Mass (kg)	62.75 ± 7.05	63.44 ± 6.39	0.69; ± 0.34	0.09

When the participants returned one year later at the same time of the season and repeated the measurements four weeks apart without any intervention, there were minimal changes in their anthropometric measurements (table 5.3).

Table 5.3 **Mean (± SD) anthropometric changes in participants of study 3 following nocebo supplementation for four weeks**

n = 4	25TT P1 (mean ± SD)	25TT P2 (mean ± SD)	Difference; ± 90%CI	*d*
Total Mass (kg)	72.43 ± 10.45	72.47 ± 11.05	0.03; ± 0.62	0.00
BMI (kg/m^2)	23.37 ± 0.88	23.37 ± 1.12	0.00; ± 0.14	-0.02
Fat Mass (kg)	10.47 ± 4.90	10.77 ± 3.95	0.30; ± 0.18	0.09
Lean Mass (kg)	89.53 ± 4.90	89.23 ± 3.95	-0.27; ± 0.16	-0.02

5.3.2 Nutrition analysis

Mean macro and relevant micro nutrient intakes over a seven day period (one typical week and one typical weekend) of the participants are presented in table 5.4. A detailed table including the COMA (1991) recommendations is presented in appendix J.

Table 5.4 Mean (± SD) nutrient intake in participants of study 2

n = 10	Mean ± SD
Energy (MJ/d)	9.6 ± 1.4
Total carbohydrates (g/d)	334 ± 75
Total fat (g/d)	60 ± 14
Protein (g/d)	93 ± 23
Alcohol (g/d)	9 ± 7
Calcium (mg/d)	757 ± 108
Vitamin D (µg/d)	2.92 ± 1.36

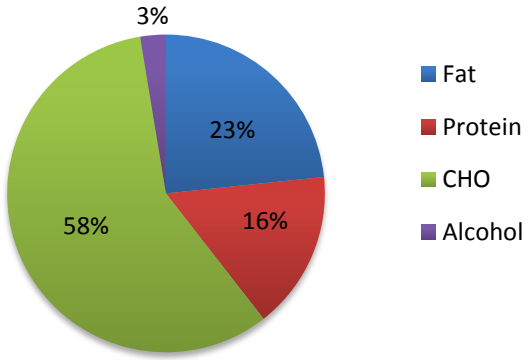


Fig. 5.2 Mean macronutrient intake in participants of study 2 as a percentage of energy intakes

5.3.3 Training logs

A summary and mean training volume and intensity as a TRIMP Score of the participants during the four weeks of supplementation is presented in Fig. 5.3. Both training volume and intensity of the participants did not increase over the supplementation period. There was no significant difference between the four weeks of training of the participants ($p = 0.22$).

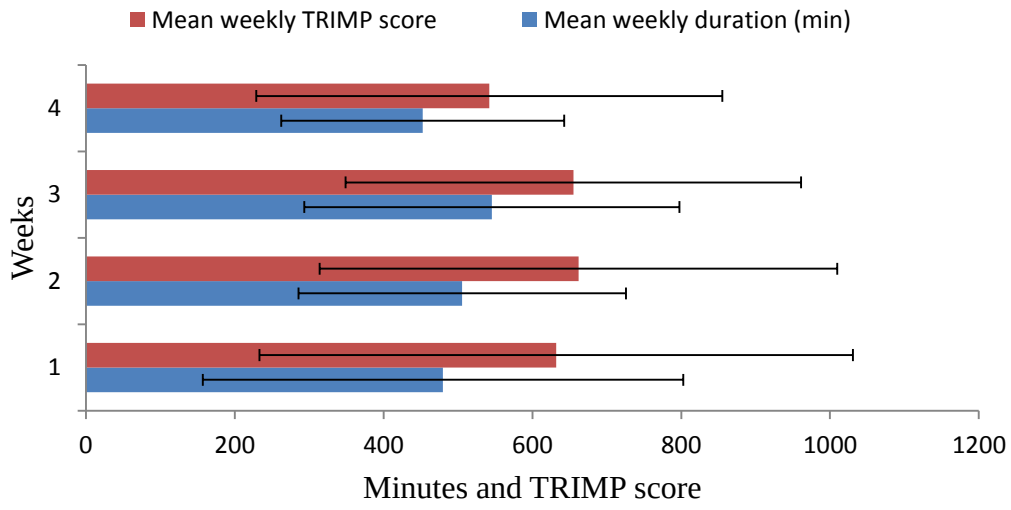


Fig. 5.3 Mean (± SD) weekly training volume (min) and intensity (TRIMP score) of the participants during the four weeks of calcium supplementation trial (n = 10)

The mean monthly monotony was calculated as 3 ± 2 and the mean monthly strain was calculated as 1882 ± 668. Examples of training logs are presented in appendix K.

The mean training volume and TRIMP score of the nocebo trials is presented in Fig. 5.4. There was no significant difference between the four weeks of the trial in the athletes training (p = 0.36).

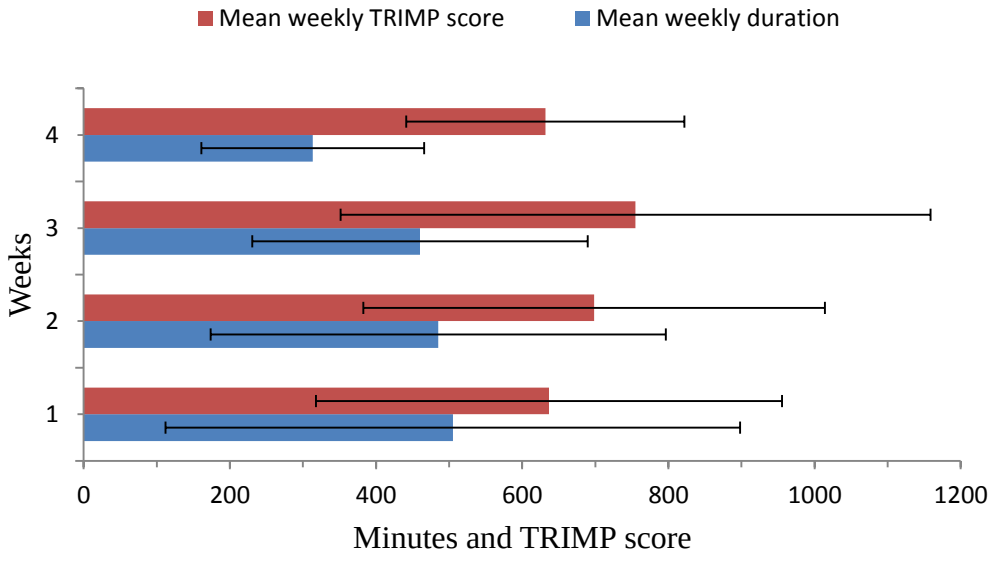


Fig. 5.4 Mean (± SD) weekly training volume (min) and intensity (TRIMP score) of the participants during the four weeks of nocebo trial (n = 4)

The mean monthly monotony in the nocebo trial was 3 ± 2 and strain was 2112 ± 517.

5.3.4 Performance variables

Mean changes in the performance variables between the two trials; before and after calcium supplementation are presented in table 5.5. All the relevant variables are discussed individually in the following sections.

Table 5.5 Mean (± SD) change in performance variables in participants of study 2, following calcium supplementation for four weeks (n = 10)

n = 10	25TTC1 (mean ± SD)	25TTC2 (mean ± SD)	% change (mean)	90%CI	*d*
Time to finish (min)	62.11 ± 2.82	60.74 ± 2.53	-2.25*	-1.52 to -1.22	-0.51
Power output (PO) (W)	236.96 ± 25.93	246.96 ± 27.66	4.05	9.1 to 10.22	*0.37*
Relative PO (W/kg)	3.23 ± 0.36	3.35 ± 0.27	3.58	0.07 to 0.17	0.38
Relative PO (W/%FFM)	*2.79 ± 0.36*	2.88 ± 0.39	3.13	0.07 to 0.11	0.24
Gross efficiency (%)	17.30 ± 1.72	19.46 ± 1.94	2.16†	2.05 to 2.27	1.17
Gross economy (W/l)	60.36 ± 6.00	67.88 ± 6.77	11.08†	7.12 to 7.92	1.17
Energy expenditure (kJ)	4885 ± 630	4614 ± 729	-5.87	-323 to 220	0.40
Heart rate (bpm)	164 ± 11	163 ± 11	-0.68	-1.26 to -0.94	-0.10
Cadence (rpm)	93.22 ± 5.06	89.84 ± 9.92	-3.76	0.4 to 5.6	-0.43

*** significant at p < 0.05 † significant at p < 0.001**

Mean changes in performance variables of the participants (n = 4) in the nocebo are presented in table 5.6 and each relevant individual variable is discussed along with the main calcium trial in the following sections.

Table 5.6 **Mean (± SD) change in performance variables in participants of study 2, following nocebo supplementation for four weeks (n = 4)**

n = 4	25TTP1 (mean ± SD)	25TTP2 (mean ± SD)	% change (mean)	90%CI	*d*
Time to finish (min)	63.55 ± 6.07	64.89 ± 5.48	2.06	0.85 to 1.83	0.23
Power output (W)	240.33 ± 54.04	230.63 ± 43.30	-4.20	-18.52 to -0.86	-0.19
Relative PO (W/kg)	3.48 ± 1.17	2.90 ± 0.41	-20	-1.3 to 0.14	0.66
Relative PO (W/%FFM)	2.74 ± 0.59	2.56 ± 0.56	-7.03	-0.21 to -0.15	0.31
Gross efficiency (%)	20.59 ± 2.42	18.23 ± 2.80	-2.36	-2.67 to -2.05	-0.90
Gross economy (W/l)	71.82 ± 8.44	63.58 ± 9.76	-12.96	-9.33 to -7.15	-0.90
Energy expenditure (kJ)	4301 ± 1330	4811 ± 1609	10.60	281 to 740	0.35
Heart rate (bpm)	164 ± 8	160 ± 9	-2.40	-5.12 to -2.48	-0.43
Cadence (rpm)	91.35± 3.50	89.80 ± 4.07	-1.73	-2.03 to -1.07	-0.39

5.3.4a Time to finish

The time to finish the 25TT after calcium supplementation for four weeks decreased significantly by a mean of 1.37 ± 0.29 min (p = 0.02) (Fig. 5.5). Most athletes decreased their time to finish with two of them increasing the time by 0.05 min and 0.06 min. The maximum decrease in time to finish was 3.79 min. Time to finish correlated with PPO significantly after calcium supplementation (r = 0.81, p = 0.03) but not before (r = -0.66, p = 0.08).

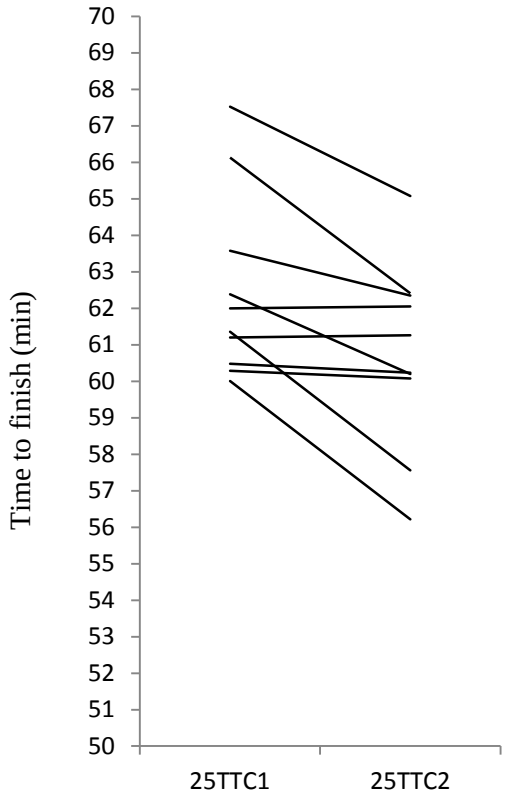


Fig. 5.5 Individual time to finish during the twenty five mile time trials before and after calcium supplementation (n = 10)

The time to finish data of individual participants (n = 4) in the nocebo trial is presented in Fig. 5.6 with a comparative graph of the same participants' data from the calcium intervention. There was a mean increase in time to finish of 1.34 ± 0.59 min (p = 0.08). With the exception one participant (0.06 min increase), all participants in the trial following calcium supplementation decreased their time to finish. The largest individual decrease was 3.79 min in the calcium trial as opposed to an increase of 0.83 min in the test in the following year.

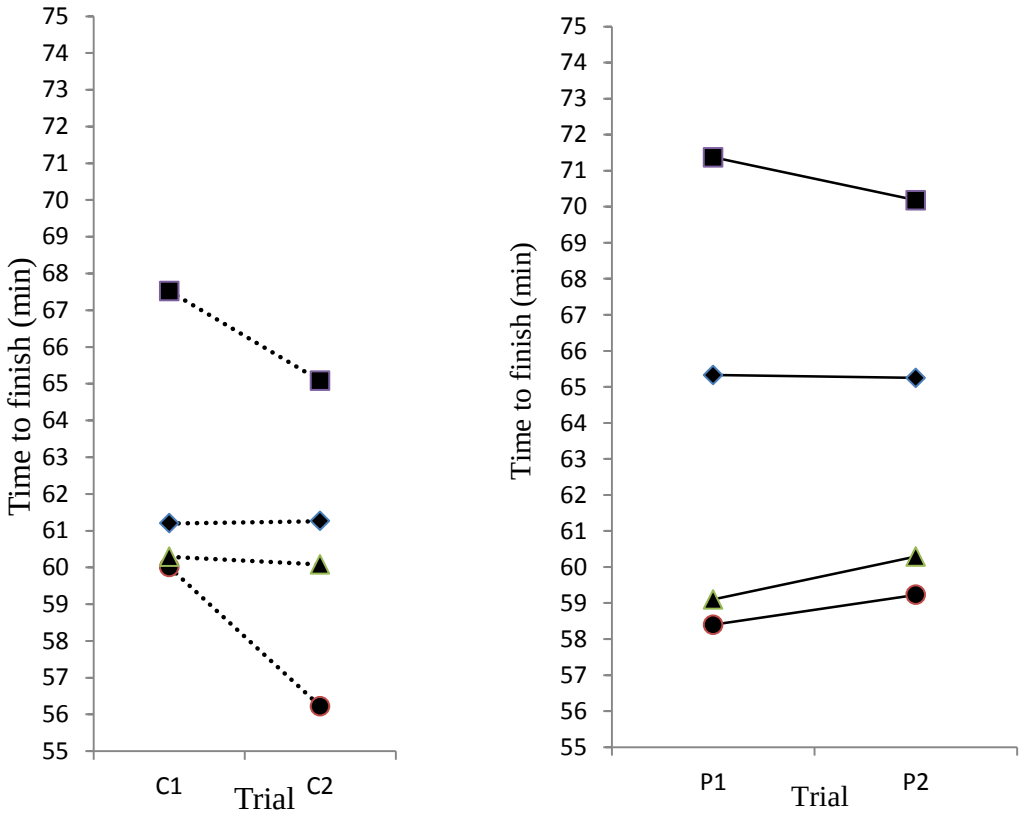


Fig. 5.6 Time to finish of the individual participants who completed both the calcium (C1 and C2) and nocebo (P1 and P2) trial (n = 4)

5.3.4b Power output

Mean power output before and after calcium supplementation is presented in Fig. 5.7. Mean power output throughout the 25TTC2 was higher compared to the trial before calcium supplementation (25TTC1) ($p = 0.06$). Power outputs were highest at the start of the exercise in both the trials and continued to decrease with time throughout the rest of the period ($p = 0.02$, main effect of time). However, during the last few minutes after the 60th min data point there were fewer participants still exercising after calcium supplementation as compared to before (25TTC1; n = 7 and 25TTC2;

n = 5). The mean power output during these last min increased (17.48 ± 20.79 W) in the 25TTC1 compared to a decrease in 25TTC2 (-7.80 ± 7.65 W) ($p = 0.12$).

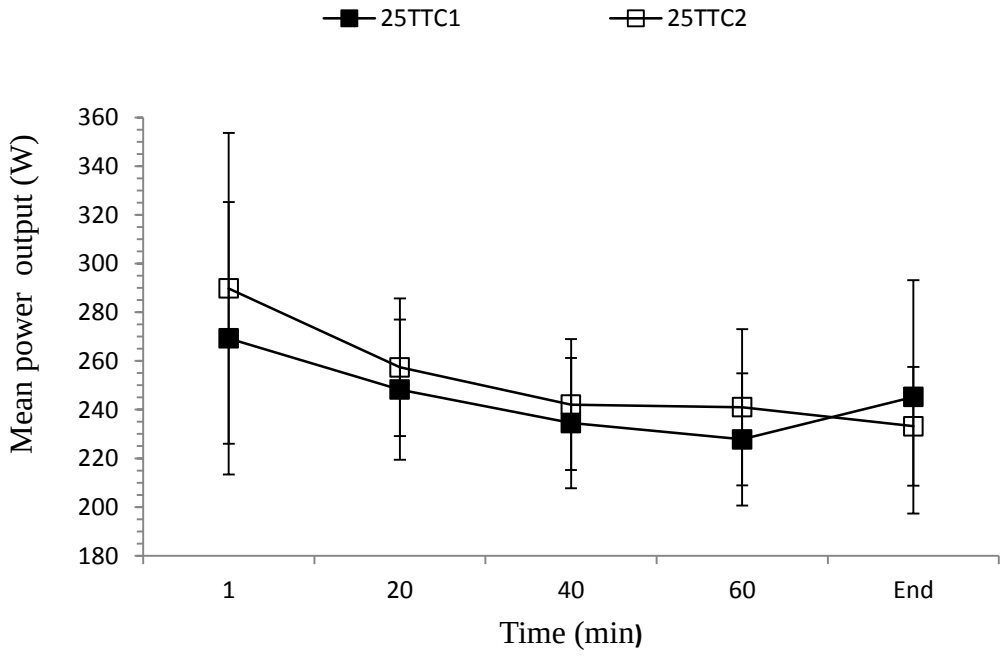


Fig. 5.7 Mean (± SD) Power output during the twenty five mile time trials before (25TTC1) and after (25TTC2) calcium supplementation (n = 10)

Mean power output in the trial a year after the calcium supplementation trial is presented in Fig. 5.8. Similar to the calcium supplementation trial mean power output was the highest at the start of the time trial and continued to decrease as the exercise session progressed in both the trials. The participants who did not finish the 25 mile ride within 60 min were the same in both the trials (n = 2 of 4), their data is averaged as the data point from 60th min to finish (End). There was a greater decrease in power output in the 25TTP2 in this data set compared to 25TTP1 (-29.20 ± 1.83 W, p = 0.12)

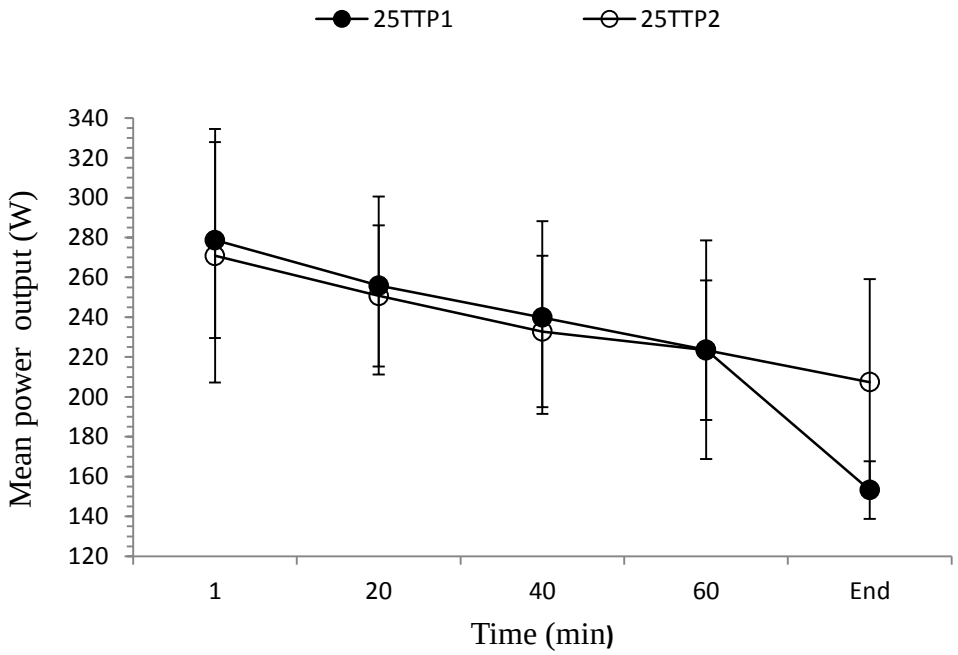


Fig. 5.8 Mean (± SD) Power output during the twenty five mile time trials in the nocebo trial before (25TTP1) and after (25TTP2) four weeks (n = 4)

5.3.4c Gas exchange

i. Oxygen consumption

Mean oxygen consumption during the trials before and after four weeks of calcium supplementation is presented in Fig. 5.9. There was a non-significant decrease in oxygen consumption after 25TTC2 (p = 0.08). Exercise values in both the trials were significantly higher than the values at the start of the trial (p = 0.01, main effect of time).

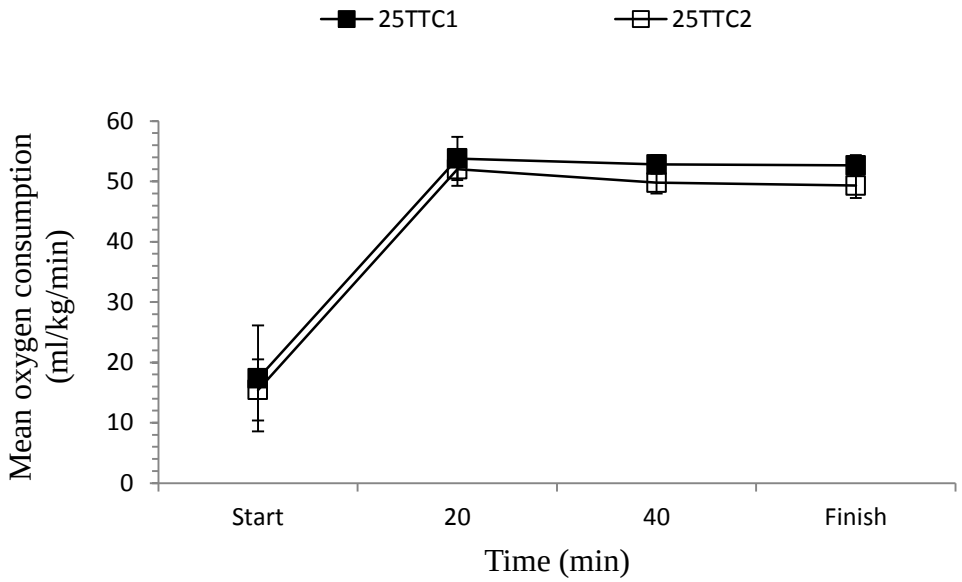


Fig. 5.9 Mean (± SD) oxygen consumption during the twenty five mile time trials before (25TTC1) and after (25TTC2) calcium supplementation (n = 10)

Mean oxygen consumption in the nocebo trial showed the opposite trend in oxygen consumption (Fig. 5.10). Mean oxygen consumption increased in 25TTP2 compared to 25TTP1 (mean increase of 5.2 ± 1.0 ml/kg/min, p = 0.10) except the start of the trials. Mean oxygen consumption was significantly different at the start of the exercise than throughout the exercise session in both the trials (p = 0.01, main effect of time).

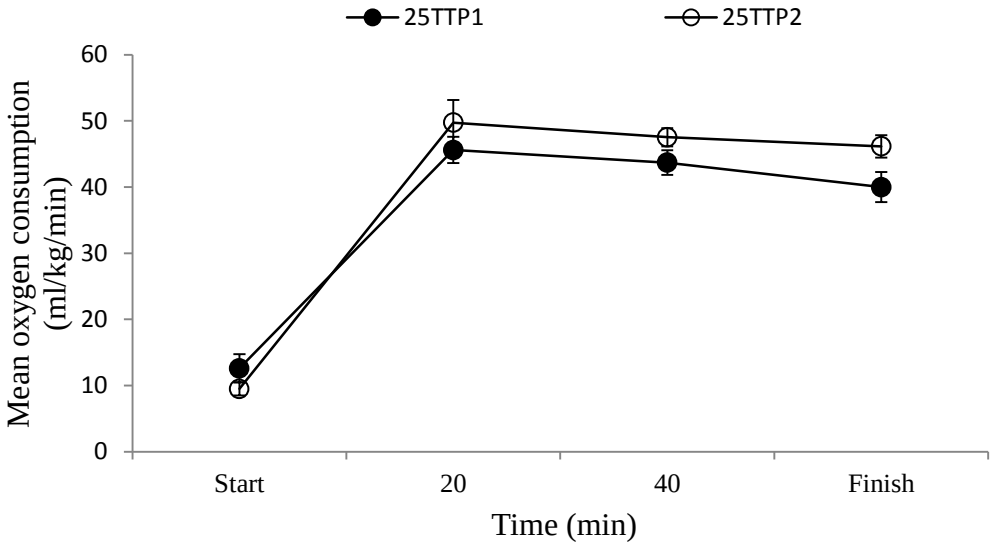


Fig. 5.10 Mean (± SD) oxygen consumption during the twenty five mile time trials in the nocebo trial before (25TTP1) and after (25TTP2) four weeks (n = 4)

ii. Respiratory exchange ratio (RER)

Mean RER was non-significantly higher in the trial following four weeks of calcium supplementation compared to before supplementation ($p = 0.08$) (Fig. 5.11). The values at the start of the exercise session were significantly higher than the rest of the period during the exercise session in both the trials ($p = 0.03$, main effect of time).

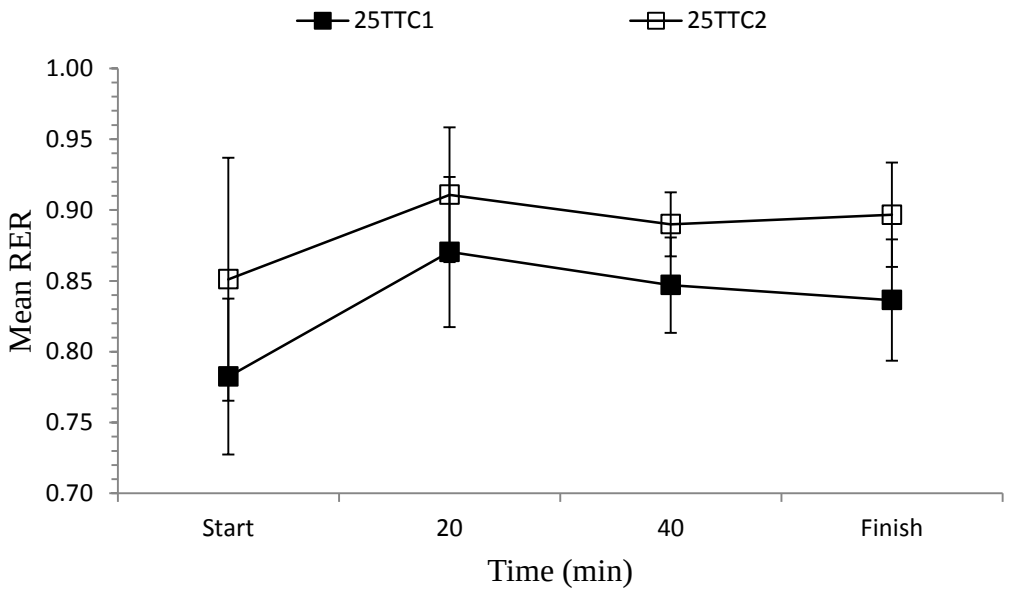


Fig. 5.11 Mean (± SD) RER during the twenty five mile time trials before (25TTC1) and after (25TTC2) calcium supplementation (n = 10)

The RER trend was reversed in the nocebo trial (Fig. 5.12). RER continued to fall in the 25TTP1 throughout the exercise session after an initial increase from the starting values ($p = 0.02$, main effect of time).

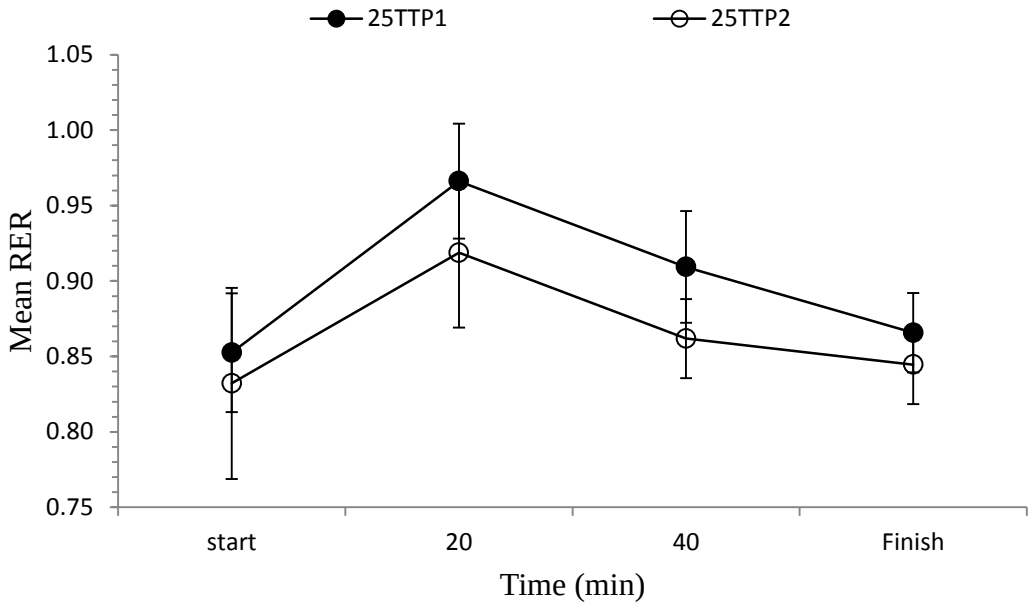


Fig. 5.12 Mean (± SD) RER during the twenty five mile time trials in the nocebo trial before (25TTP1) and after (25TTP2) four weeks (n = 4)

5.3.4d Substrate metabolism

Mean total energy expenditure (TEE) and the percentage carbohydrate and fat are depicted in Fig. 5.13. Mean TEE was marginally higher in 25TTC1 (4885 ± 630 kJ) compared to 25TTC2 (4614 ± 729 kJ) ($p = 0.22$) and the mean total contribution of carbohydrate was 15.11 ± 1.93 % higher in 25TTC2 compared to the trial before calcium supplementation. Proportionately the contribution of fat decreased in 25TTC2 15.11 ± 1.93 % compared to 25TTC1. These results were not statistically significant.

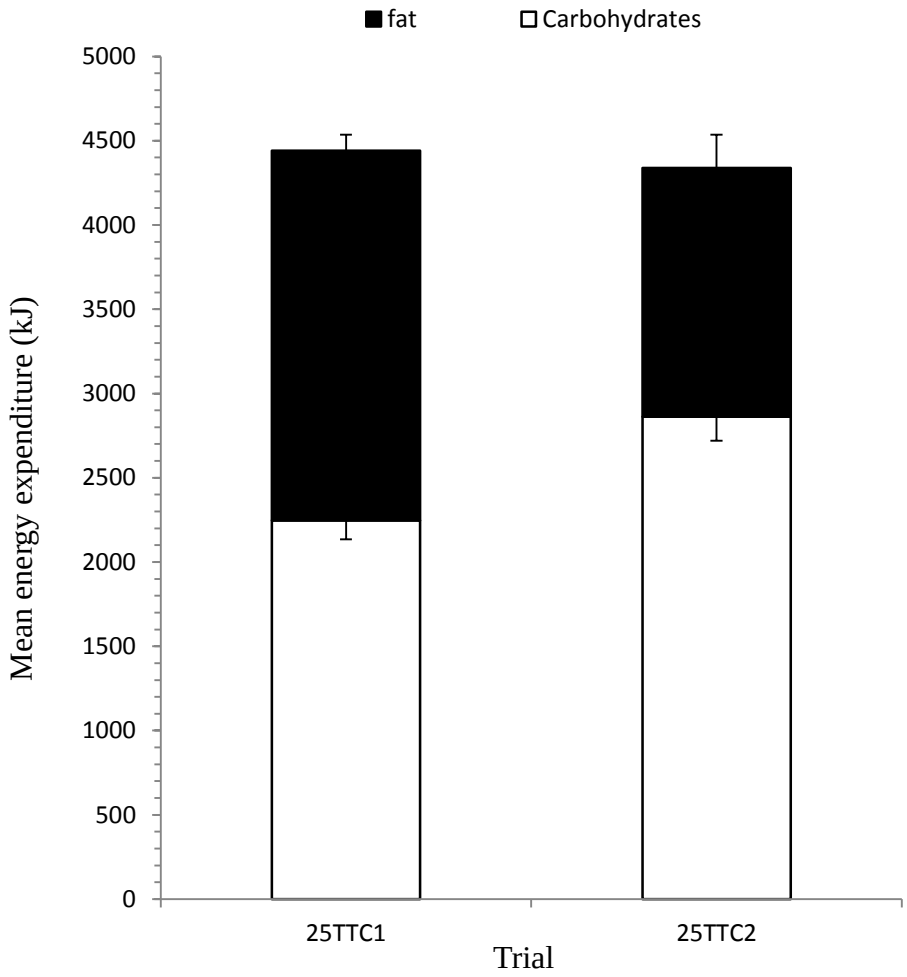


Fig. 5.13 Comparison of mean total energy expenditure and percentage of the energy derived from carbohydrates (white) and fat (black) in twenty five mile time trials

5.3.4e Gross efficiency

Athletes produced higher power at a given rate of oxygen consumption after calcium supplementation ($p < 0.001$). This trend was observed across the range of oxygen consumption values during the twenty five mile time trials.

The mean gross efficiency of the participants improved throughout the trial after calcium supplementation than before ($p = 0.001$, main effect of supplementation) (Fig. 5.14). Gross efficiency was highest at the start of the exercise session for both the trials ($p = 0.01$, main effect of time) and continued to decrease in the trial before calcium supplementation. Mean gross economy of the athletes was a mean of 60.36 ± 6.00 W/l in 25TTC1 and 67.88 ± 6.77 W/l during 25TTC2 ($p = 0.001$, main effect of supplementation).

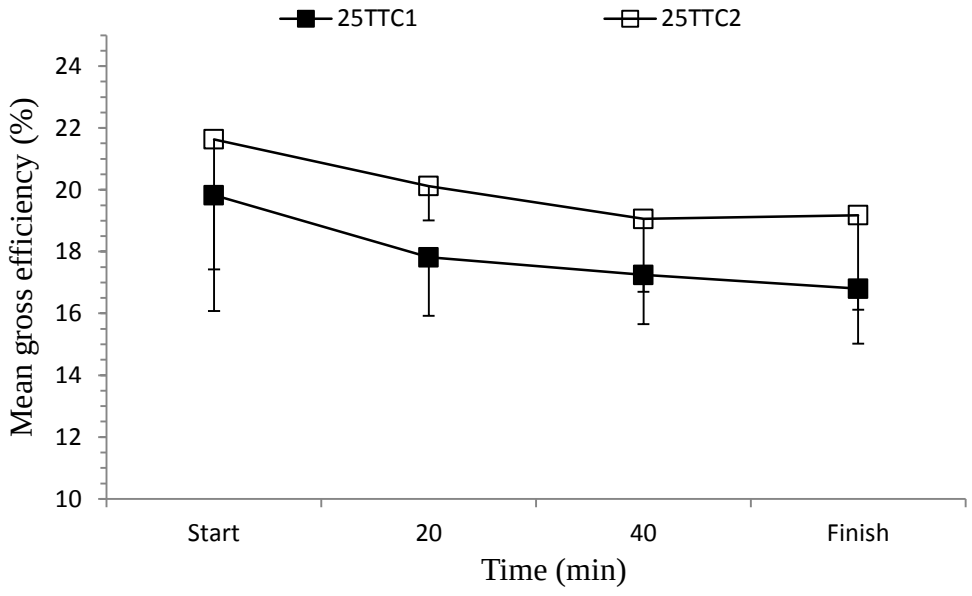


Fig. 5.14 Mean (± SD) gross efficiency during the twenty five mile time trials before (25TTC1) and after (25TTC2) calcium supplementation (n = 10)

Mean gross efficiency in the nocebo trial is presented in Fig. 5.15. Gross efficiency was lower in 25TTP2 compared to the trial prior 25TTP1 (-2.36 ± 0.38, p = 0.07). Mean GE was higher at the start of the exercise to all other time points and was lowest at the end of the exercise period in both the trials.

Gross economy of the athletes was a mean of 71.82 ± 8.44 W/l in 25TTP1 and 63.58 ± 9.76 W/l in 25TTP2 ($p < 0.001$, main effect of supplementation).

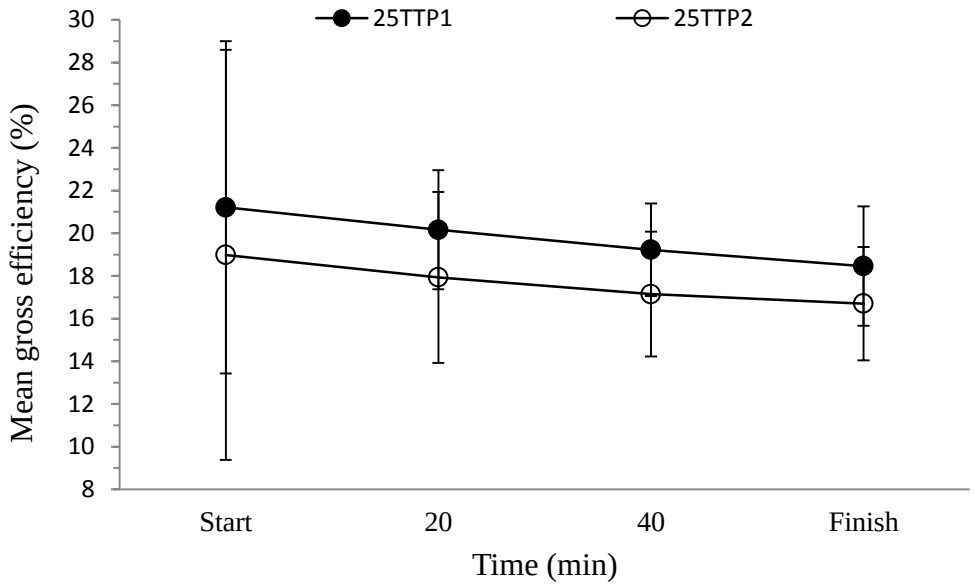


Fig. 5.15 Mean (± SD) gross efficiency during the twenty five mile time trials in the nocebo trial before (25TTP1) and after (25TTP2) four weeks (n = 4)

5.3.4f Haematological variables

i. Plasma glucose

Mean plasma glucose in the trials before and after calcium supplementation is presented in Fig. 5.16. There were no significant differences between the plasma glucose values in both the trials ($p = 0.23$).

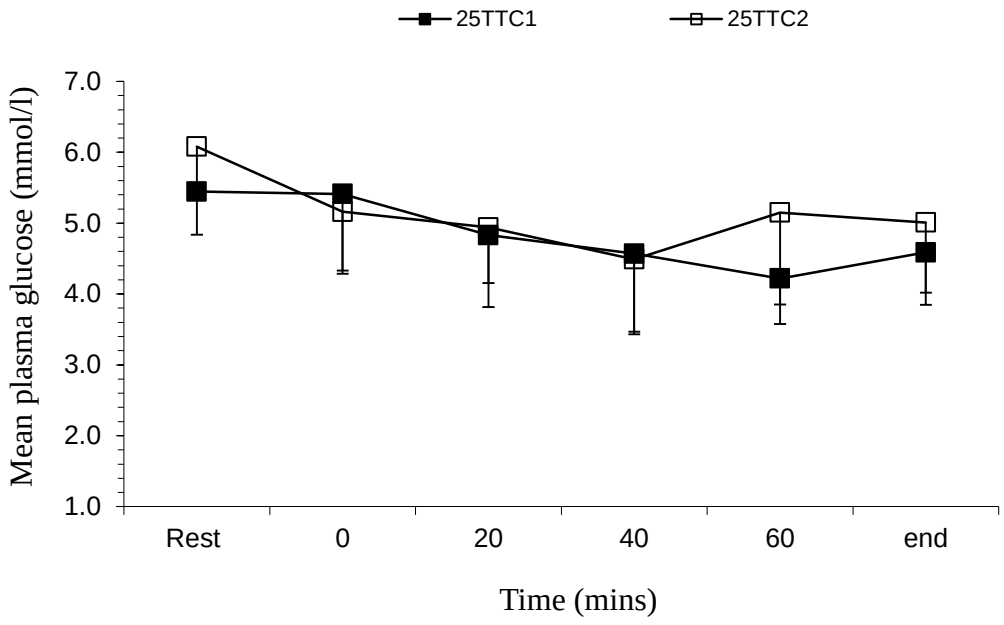


Fig. 5.16 Mean (± SD) plasma glucose during the twenty five mile time trials before (25TTC1) and after (25TTC2) calcium supplementation (n = 10)

ii. Plasma lactate

Mean plasma lactate was was not significantly different in both the trials (Fig. 5.17). However it was non-significantly higher in the 25TTC2 after forty min until the end of the exercise ($p = 0.12$).

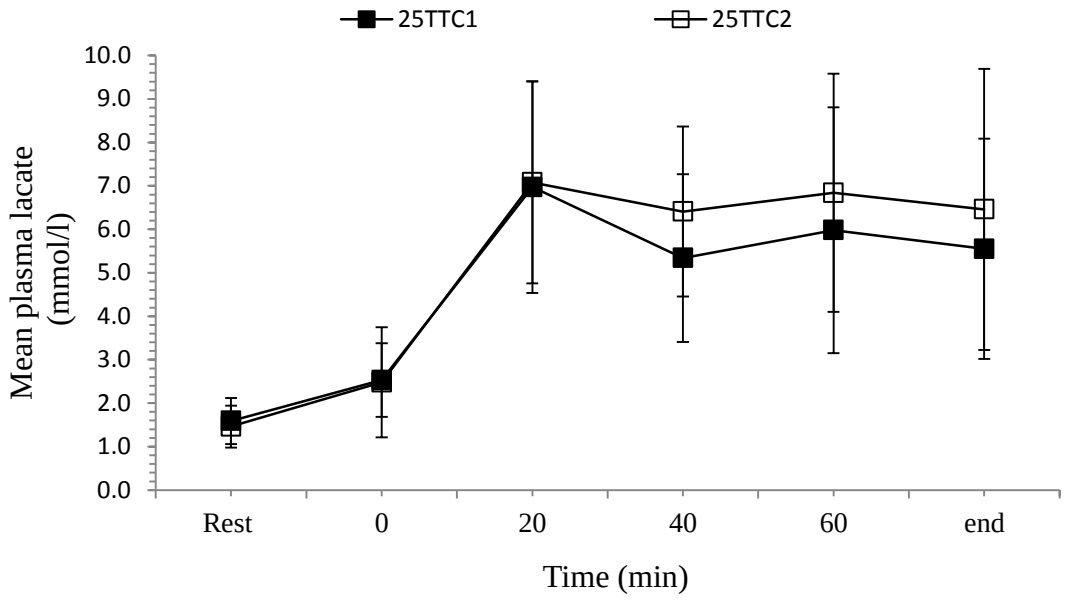


Fig. 5.17 Mean (± SD) plasma lactate during the twenty five mile time trials before (25TTC1) and after (25TTC2) calcium supplementation (n = 10)

iii. Plasma non-esterified fatty acids (NEFA)

Mean plasma NEFA values from both the trials, before and after calcium supplementation are presented in Fig. 5.18. Mean plasma NEFA were higher throughout rest and exercise in the 25TTC2 trial (mean difference 0.031 ± 0.002, $p > 0.08$, main effect of supplementation). There was a significant difference in the values at rest and at the end of exercise in both the trials ($p = 0.02$, main effect of time).

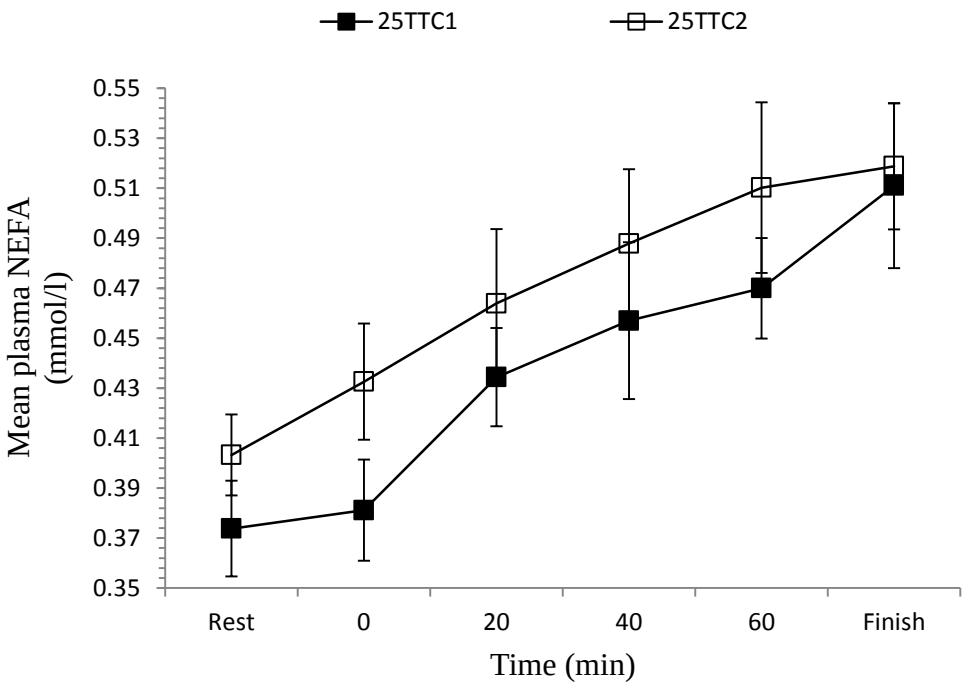


Fig. 5.18 Mean (± SD) NEFA during the twenty five mile time trials before (25TTC1) and after (25TTC2) calcium supplementation (n = 10)

5.4 Discussion

5.4.1 Performance variables

The principle aim of this study was to determine the effect of calcium supplementation of four weeks on endurance cycling performance. The main findings from this study indicate that increasing dietary calcium has a beneficial effect on cycling performance via mechanisms that are different to manimuplation of fat oxidation rates. Athletes completed the 25 TT in a shorter time (-1.37 ± 0.29 min) after calcium supplementation. This was achieved via an increased power output throughout the exercise period. Increased power output was maintained despite lower mean oxygen consumption. Thus GE values were significantly higher in 25TTC2 compared 25TTC1 (2.16 ± 0.22 %, $p < 0.001$). These results can be considered as meaningful for an athlete because the change was more than 1% (Currell and Jeukendrup, 2008, Paton and Hopkins, 2001). GE has been suggested by Coyle et al. (1992) to be a key determinant of endurance performance. GE in other publications (Sassi et al., 2008, Hopker et al., 2009) are in the similar range for the training status and $\dot{V}O_{2peak}$ values of the participants as this study.

GE in cycling is reported to be influenced by numerous factors such as but not limited to; muscle fibre type (Coyle et al., 1992), body position (Ashe et al., 2003), altitude exposure (Hahn and Gore, 2001, Gore et al., 2001), years of training (Hopker et al., 2007, Coyle, 1999), uphill riding and standing or sitting positions (Millet et al., 2002), cadence and pedalling technique (Cannon et al., 2007). Improvements in GE have been reported as a consequence of training (Hawley and Stepto, 2001); with (Luttrell and Potteiger, 2003) or without (Williams et al., 2009) specialised equipment such as power cranks. However, apart from different training

regimes which may have beneficial physiological and biomechanical adaptations, not many other avenues to improve GE have been explored in the literature. This study is the first to show that calcium supplementation has a beneficial effect on GE in cycling.

A small change in GE can correspond to marked improvements in performance. Jeukendrup et al. (2000) have estimated that for an elite cyclist with a mass of 70 kg and a 1 hr sustainable power of 400 W, a 1% improvement in GE equates to a 48 sec improvement in a 40 km time trial. During this trial the mean power sustained over 1 hr was ~250 W (246.96 ± 27.66 W) after calcium supplementation and a reported increase in GE of 2.16%, which amounts to a 63 sec improvement in time to finish with 1% improvement in GE. This is a larger improvement than the calculations in Jeukendrup et al. (2000) considering the cyclists in this study sustained a lower power output over 1 hr, and the mean body mass of these athletes was slightly higher (73.8 ± 9.31 kg) than the calculations based on 70 kg body mass in Jeukendrup et al. (2000). However interestingly, when these calculations were made via modelling software, the same authors have quoted a 63 sec improvement with a 1% improvement in efficiency if the rider sustains ~300 W over the 40 km (Moseley and Jeukendrup, 2000). They (Moseley and Jeukendrup, 2000) also state that this time gain would be even greater in less skilled riders. This is more consistent with the data from this study since all the participants were non elite but well-trained and competitive at the club level (mean $\dot{V}O_{2peak}$ 62.70 ± 6.43 ml/kg/min and $W_{peak\ (aerobic)}$ 350 ± 42 W).

In addition, seasonal variations and improvement in GE during pre season time (Hopker et al., 2009) were controlled for in this study by conducting all the tests

between September and December. This corresponds with the end of the racing season and the beginning of low intensity moderate volume training in the winter months. GE during this period is reported to decrease due to drop in training intensity (White et al., 1982a). Concurring with their results, the subsequent trial in this study which was conducted at the same time the following year resulted in a mean decrease in GE (-2.36 ± 0.38%) in four weeks with similar training and nutrition status of the returning participants (n = 4). Therefore the increase in GE in the calcium trial could at least in part be explained as a result of increase in dietary calcium intakes of these athletes.

Coyle et al. (1991) has demonstrated that actual performance of elite national class and good state class cyclist in a 40 km time trial highly correlates with average absolute power during a one hour simulated laboratory test (r = -0.88). As argued in Chapter 3 these laboratory simulations reflect actual demands of such cycling events reasonably well. It has been reported that in elite cyclists completing a 40 km time trial in ~54 min, power output at LT was 311 W (Coyle et al., 1991). The mean power output at LT in this study was 238 ± 16 W which achieved a time to finish of ~60+ min for a similar distance. Since the athletes in the present study were club level competitive riders these values concur with Coyle et al. (1999) and others (Moseley et al., 2004, Hopker et al., 2009). Maintaining a comparative higher power output over the period of ~60 min would require in part the attenuation of mechanisms that may involve fatigue of the skeletal muscle. Various studies looking at force decrease during fatigue have reported increased myofibrillar calcium sensitivity and increased resting $_{i}[Ca^{2+}]$ (Chin et al., 1997) which occur due to metabolic changes that may in part be related to decreased glycogen content in muscle at exercise intensities of 60 - 80 $\%\dot{V}O_{2max.}$ The athletes in this study rode at a

mean 70.88 ± 4.80 % W_{peak} after calcium supplementation compared to 67.97 ± 4.11 % W_{peak} at baseline. Therefore it could be argued that calcium supplementation may have been responsible in this sustained increased power output.

Dietary calcium intake has a profound impact on calcium homeostasis. Research has shown that depressed calcium intake can substantially increase $_{i}[Ca^{2+}]$ levels in human adipocytes (Xue et al., 2001) and skeletal muscle (via restricted energy intake ~400 Kcal/d) (Russell et al., 1983). Experiments conducted by Barclay (1996) using mouse fast twitch extensor digitorum longus (EDL) muscle and slow twitch soleus using fatigue protocols (thirty isometric tetani) decreased the efficiency of energy conversion by the crossbridges and also reduced the curvature of the force velocity relationship. These fatigue signals seem to be amplified in an increased $_{i}[Ca^{2+}]$ environment (Cairns and Lindinger, 2008). In skeletal muscle, decreased creatine phosphate reduces the available energy for the calcium efflux and sequestration into the sarcoplasmic reticulum (Russell et al., 1984). The correlation of muscle relaxation with free energy of adenosine triphosphate (ATP) hydrolysis suggest that a decrease in relaxation rate and an increase in fatigue may result from slowing of pumps transferring free calcium into the sarcoplasmic reticulum (Dawson et al., 1980), and a considerable depletion in extracellular calcium levels in the transverse tubules of the skeletal muscle sarcolemma may contribute to fatigue (reduced force output) (Cairns et al., 1998).

These mechanisms leading to fatigue are possibly attenuated by a change in calcium homeostasis via increased extracellular availability of calcium during a period of high intake. When dietary calcium is increased, it reduces entry of calcium into the intracellular compartments via a calcitriol and parathyroid mediated enzymatic

cascade and the resulting higher extracellular calcium levels compared to the intracellular stores (Xue et al., 2001). It is possible that four weeks of calcium supplementation attenuated these fatigue mechanisms in the participants allowing them to ride at a higher power output with no further increase oxygen consumption required. However, this is only possible if the glycogen stores are well replenished (Romijn et al., 1993) as the increased power production would in turn require a higher proportion of carbohydrate availability (Sherman et al., 1991). Also, specific carbohydrate availability is implicated in muscular excitability (Russell et al., 1983, 1984). For example glucose administration partially restored force during late fatigue in muscles stimulated *in-situ* (Marcil et al., 2005) and in exercising humans (Stewart et al., 2007). This is in part explained by the amplified effects of muscle glycogen depletion on potassium (K^+) induced depolarisation to reduce excitability during exercise (Cairns and Lindinger, 2008). Increased extracellular calcium antagonises this K^+ depletion (Cairns and Lindinger, 2008) to further alleviate muscular fatigue responses during submaximal exercise.

Another effect of increased metabolic activity in working muscle is the production of reactive oxygen species (ROS) (Allen et al., 2008) which may interact with a diminished K^+ gradient to further impair contraction during exercise. Increased $_i[Ca^{2+}]$ due to a decrease in dietary calcium intake increases ROS production in all cells (Zemel and Sun, 2008) and this coupled with exercise induced ROS detrimental effect on exercise performance can further contribute towards fatigue.

5.4.2 Body mass and composition

One of the key physiological variables associated with cycling performance is body mass, which increases the energy cost of acceleration, rolling resistance and the projected frontal area of the cyclist (and hence air resistance) (Jeukendrup et al., 2000). Olds et al. (1995) have estimated that an extra fat mass of 2 kg would increase a 4000 m individual pursuit time by 1.5 sec or a distance of ~20 m. In the one hour record Bassett et al. (1999) have suggested that 60% improvement in performance could be attributed to advances in the aerodynamics of the bike, rider position etc. and 40% from the ability of the rider to sustain a high power output. Indeed when PO was accounted for the body composition changes, the cyclists were riding at an average higher power output per kg FFM in the trial following calcium supplementation compared to baseline. FFM after calcium supplementation increased by 0.81 ± 0.15 % (0.69 ± 0.66 kg) and reduced fat mass by 0.58 ± 0.22 kg compared to the baseline measurements and therefore the decreased time to finish the twenty five mile time trial and the subsequent increased power output seems to be related to increase in fat free mass and/or the subsequent decrease in fat mass in our trials. The importance of contribution of optimal levels of fat free mass in performance over total mass has been highlighted in many studies looking at performance in cycling (White et al., 1982a, Housh et al., White et al., 1982b). Thus, even the small change in body composition during the trial could have been an important ergogenic aspect of the calcium intervention.

5.4.3 Plasma lactate

Mean plasma lactate even though statistically non-significant, showed an increase in 25TTC2 compared to 25TTC1. It is possible that this may have been an important outcome due to the supplementation. The accumulation of lactate and especially associated photons, both intracellular and extracellular compartments have long been thought to contribute to the fatigue of high intensity submaximal exercise. However, Robergs et al. (2004) have beautifully explained the case of how lactate production retards, not causes acidosis. Neilson and co workers (2001) have also demonstrated that extracellular lactic acid accumulation has a protective effect on muscle excitability (on K^+ induced force depression and impairment of calcium release from the sarcoplasmic reticulum) and these effects work in conjunction with elevated adrenaline on stabilising membrane excitability (de Paoli et al., 2007). This study was performed in isolated muscle fibres and it remains unknown if these mechanisms that operate under controlled conditions *in vitro* occur to a similar degree in complex *in-vivo* systems. However, the data from this investigation certainly inch towards supporting the hypothesis that increased lactate production may indeed have a performance enhancing effect by way of maintaining and even increasing power output of the cycling time trial via improving membrane contractibility.

In addition, it is also possible that the lactate anions provide oxidizable substrate during long duration exercise, this may have aided the performance of the time trial after calcium supplementation, as lactic acid (lactate and associated proton) clearance through oxidation and gluconeogenesis during exercise has been thought to provide a beneficial and alkalizing effect on blood pH (Brooks, 2010, Gladden, 2004). In addition, lactate accumulation also seems to be associated with redox

(oxidation – reduction) potential that may help sustain intramuscular ATP production and thus performance (Sahlin et al., 1987).

One of the interesting effects of lactic acid production is its effects on ionised calcium fractions in blood. Hydrogen ions release calcium ions from those bound to protein and lactate chelates this free calcium ions (Toffaletti and Abrams, 1989). In the absence of any direct data from submaximal exercise, a study by (Ljunghall et al., 1985) reported a net reduction in plasma ionised calcium content during an experiment with isokenetic one legged exercise. They have attributed this decrease to formation of calcium – lactate complex. This concurrent decrease in plasma ionised calcium would normally stimulate parathyroid hormone to mobilise calcium from the bone. However, it is possible that calcium supplementation may have some protective effect in maintaining the ionised calcium levels during exercise without a subsequent rise in iPTH that may in turn affect the lipolytic and associated metabolic cascade (Zemel, 2002) to impair performance. It is not possible to comment any further on iPTH responses in this study as they were not measured.

These mechanisms perhaps explain to a certain degree the performance enhancing effect of plasma lactate combined with calcium supplementation; however they do not explain why lactate production was increased after four weeks of calcium supplementation in the same exercise compared to the trial without supplementation. One way to look at the data is that since fatigue may have been attenuated in the trial after supplementation of calcium via multiple mechanisms, it allowed the athletes to perform at a relatively higher intensity (increased power output) thus displaying the well established linear relationship between plasma lactate values and exercise intensity (Coyle, 1995a).

5.4.4 Substrate metabolism

There was no significant difference in the total energy expenditure between the two trials. This concurs with various other studies that have looked at the influence of dietary calcium on energy expenditure in humans (Melanson et al., 2003, Jacobsen et al., 2005, Teegarden et al., 2008) or mice (Papakonstantinou et al., 2003). But it is the reverse of what was observed in study 1, where the athletes were required to cycle at a fixed intensity for 60 min before and after calcium supplementation. In fact, metabolic energy expenditure calculated as a product of oxygen consumption was slightly lower (~125 kJ) in 25TTC2. Mean oxygen consumption decreased with a concurrent increase in RER values indicating that the intensity of exercise performed was higher in the trial following calcium supplementation with a concurrent increase in power output. The percentage contribution of fat as a substrate decreased in the trial after calcium supplementation by ~15%. The decreased rate of fat oxidation may not be a reflection on the effect of calcium supplementation, but an artefact of increased intensity of exercise in the 25TTC2 which the athletes were able to sustain perhaps via the metabolic pathways discussed earlier. Since the intensity of exercise did not remain the same it would not be possible to make any direct comparisons on the influence of dietary calcium on fat oxidation in time trials where exercise intensity is regulated by the athlete in order to achieve the best time-to-completion of a set distance.

Studies looking at specifically fat oxidation effect of calcium supplementation in non exercising population have reported positive correlations between calcium intakes and fat oxidation rates. Melanson et al. (2003) have reported positive correlations between acute calcium intake (over a wide range of intakes within a 24 hour period) and fat oxidation rates in that 24 hour period. Interestingly, this correlation

disappears with habitual calcium intake (dairy and total calcium). But several longitudinal studies have reported increases in fat oxidation rates as a result of increased calcium intakes. A 12 week intervention with 900 mg/d calcium supplementation in overweight women (75.5 ± 9.6 kg) increased fat oxidation by 1.5 ± 0.6 g/h (Teegarden et al., 2008). An even longer intervention of one year in young women reported an increase in fat oxidation (Gunther et al., 2005).

However, the blood indices indicate a higher NEFA availability in blood during the trial after calcium supplementation, indicating an increased rate of lipolysis. It is therefore possible that this study did not see an increase in fat oxidation rates as the intensity of the exercise was not in the range of maximum fat oxidation (Horowitz and Klein, 2000b) and therefore the available NEFA may have either re-esterified (Romijn et al., 1995) or substrate oxidation rates would have altered post the exercise session (Romijn et al., 1993). Recovery RER or NEFA was not measured in this study to make further inferences on this point. However, Men'shikov (2004) have reported a multiple correlation between after load plasma free NEFAs, changes in plasma ionised calcium and oxygen consumption ($r = 0.72$, $p < 0.05$).

It is also possible that the increased lipolytic rate may be a function of higher exercise intensity (Horowitz and Klein, 2000b) rather than any influence of calcium supplementation. Since increased lipolysis during exercise is directly correlated to the catecholamine response (Arner et al., 1990, Coppack et al., 1994) and increased dietary calcium has shown to influence an increase in catecholamine response (Izawa and Komabayashi, 1994). Further supporting that premise Kozyreva (1983) found that adaptation to low temperatures is associated with a decrease in blood calcium and an increased sensitivity of the β-adrenoreceptors to norepinephrine. But, there are conflicting reports in the literature with regards to the effect of dietary calcium

on lipolysis per se. Some studies have reported theoretical mechanistic (Xue et al., 2001) and physiological observations in humans (Parikh and Yanovski, 2003, Zemel, 2003) and animals (Shi et al., 2001a, Zemel et al., 1995) implicating a positive correlation with dietary calcium and increased lipolysis. Jacobsen et al. (2005) have reported an increase in NEFA levels in overweight (26.5 ± 2 kg/m2) male (n = 8) and female (n = 2) after one week of high calcium (1800 mg/d) with normal protein intake (15%EI) of 66 ± 212 µmol/l. In a study by Cummings et al. (2006) calcium intake (575 calcium citrate mg in breakfast) acutely stimulated post prandial fat oxidation along with a decreased suppression of NEFA independent of insulin action. However, Lorenzen et al. (2007) reported suppressed post prandial lipidemia only with a meal high in dairy calcium (793 mg) and no such effect with supplemental (850 mg elemental calcium from carbonate) dose. In another study five weeks of calcium phosphate (800 mg/d) intervention did not show an effect on fat metabolism or energy expenditure in resting conditions or caffeine and epinephrine stimulation (Bortolotti et al., 2008). It is difficult to make outright comparisons with the results of these studies however, because the demographics of the sample were mainly overweight or obese populations and these blood indices reflect a resting fat metabolism state rather than an intense exercise period.

Hormonal changes such as iPTH and subsequently 1,25 $(OH)_2D_3$ has been shown to have immediate effects on lipolysis (Shi et al., 2001b, Zemel et al., 2000, Xue et al., 2001). A seven day high calcium diet (1259 ± 9 mg/d) intervention has shown significant decrease in plasma 1,25 $(OH)_2D_3$ (175 ± 16 to 138 ± 15 pmol/l) (Boon et al., 2005). Subsequently, they also reported a significant decrease in adipose tissue NEFA synthase mRNA expression in a group of lean people consuming a diet high in dairy calcium, but this was only at an intake of 2500 mg/d of calcium and they so

no effect with a more realistic 1200 mg/d calcium intake (Boon et al., 2007). iPTH has been shown to increase during intense (Kristoffersson et al., 1995) and submaximal exercise (Ljunghall et al., 1986a, Ljunghall et al., 1988) perhaps through adrenergic stimuli (Rasmussen, 1971). Also, *in-vitro* and *in-vivo* experiments have demonstrated that an increase in NEFA causes complex binding of free calcium ions as well as enhanced binding of calcium to albumin (Zaloga et al., 1987). This reduction in the ionic calcium levels due to submaximal exercise stimulates iPTH release from the thyroid gland (Ljunghall et al., 1986a) which in turn may stimulate calcium release from the bone. Plasma ionic calcium concentrations were not directly measured in this study, however, Zaloga et al. (1987) have demonstrated that this binding of NEFA to calcium ions has a net effect of significant reduction in plasma ionic concentrations, but total calcium concentrations (which include complex and albumin bound fractions) are unchanged. However, Salvesen et al. (1994) and Vora et al. (1983) have reported an increase in plasma total calcium and ionised calcium after a treadmill exercise session of moderate intensity. Without directly measuring plasma calcium levels it would be speculative to infer the effects of supplementation on the hormones related to changes in plasma calcium concentrations. But it is possible that the higher NEFA levels seen after calcium supplementation of four weeks may have been a consequence of the changes in hormonal milieu due to the supplementation or may just be an effect of the increased intensity of cycling sustained by the athletes who may have stimulated greater lipolytic response or even hemoconcentration.

There was no difference in mean plasma glucose values between the two trials in the first forty minutes. However, in the last 20 min of the exercise plasma glucose increased by 0.93 ± 0.66 mmol/l in 25TTC2 compared to 25TTC1. As the mean

power output was higher in the trial after calcium supplementation this increase could have been a result of increased metabolic activity via adrenergic stimulation (Exton et al., 1972). Even though calcium has been implicated in the release of insulin (Krahl, 1966, Clausen, 1979) and chelation of $_{i[Ca2+]}$can prevent insulin from stimulating glucose transport and glucose oxidation in adipocytes (Pershadsingh et al., 1987) and cardiac muscle (Cheng et al., 1993) . At most, speculatively it is possible that plasma glucose increase in the last 20 min of exercise in the trial following calcium supplementation could be related to a similar action of calcium supplementation in skeletal muscle as well.

5.5 Conclusions

The results from this study suggest that calcium is beneficial to endurance type events. There has been a meaningful reduction in time to complete the time trial with a concurrent increase in gross efficiency and economy of the riders (Thus supporting hypothesis iii). This was achieved via increases in power output capacity and perhaps an involvement of metabolic substrates that may have been altered due to dietary calcium. This study shows that even though calcium may improve rate of lipolysis in exercising humans, the oxidation of substrates is closely dictated by the relative intensity of the exercise (Coyle et al., 1997, Romijn et al., 1993, Romijn et al., 1995). This is an important point for athletes, coaches and sports nutritionists to consider whilst designing nutritional and training strategies to maximise adaptations to fat oxidation via novel approaches such as increase in intake of dietary calcium.

With regards to the body composition of the participants, there was no statistically significant decrease in fat mass (thus refuting hypothesis iv). However, trends

certainly continue to support the hypothesis of increased calcium intake with a reduction in fat mass (Zemel, 2003, Zemel et al., 2008, Zemel et al., 2000).

Nonetheless, the role of calcium in improving the athletes' ability to maintain higher power outputs over 60 min periods seem to be an avenue that has potential for further research. However, considering the potential effects of calcium on power output, it is important to test this hypothesis further with a higher exercise intensity protocol to study if the demands of higher power output can further augment the metabolic consequences that occur as a result of calcium supplementation.

CHAPTER 6

EFFECTS OF CALCIUM SUPPLEMENTATION ON PERFORMANCE OF A TEN MILE CYCLING TIME TRIAL

6.1 Introduction

The previous study indicated that calcium supplementation improves 25TT performance. The main findings suggested that increased power output and the corresponding improvements in time-to-completion without a concurrent increase in oxygen uptake were the responsible factors in meaningful improvements in performance. The present study was conducted to quantify the range of effect of calcium supplementation on performance. A ten mile time trial (10TT) a standard race duration, is markedly different metabolic interactions of the substrates from that of an hour long (25TT) endurance event. Thus this study will help identify the role of calcium in performance where fat oxidation is not the primary source of energy.

Increased dietary calcium seems to play a role in increasing the concentration of NEFA in the plasma (Zemel, 2004, Zemel, 2003, Xue et al., 1998). However the fat oxidation rates were not concurrently increased in the 25TT indicating other mechanisms of ergogenicity. Increased power output might have been one of the important mechanisms through which calcium exerts an ergogenic effect. A 10TT

would require a higher mean PO over the duration of the exercise compared to the 25TT therefore allowing the observation of the range of this effect of calcium supplementation on endurance performance. Thus, if the role of calcium in performance enhancement is broader than just that of change in the availability of substrate metabolism this would be more pronounced in the 10TT. Factors such as attenuation of fatigue (Cairns et al., 1998) and other related effects such as changes in the ionic state of the extra cellular and intracellular compartments, that play a larger role in more intense exercise (Nummela et al., 2008, Allen et al., 2008b, Robergs et al., 2004) will be highlighted through improvement in performance.

Hence, the aim of this experimental trial was to study the effects calcium supplementation on performance during a ten mile cycling time trial. This would be achieved by investigating its effects on power output and improvement in gross efficiency of the athletes.

6.1.1 Hypotheses

v. It is hypothesised that calcium supplementation of 1000 mg/day for four weeks improves cycling performance via improvements in power output and gross efficiency of athletes.

vi. It is hypothesised that calcium supplementation of 1000 mg/day for four weeks does not improve performance in a 10 mile time trial via changes in substrate availability.

6.2 Methods

6. 2.1 Participants

Ten male healthy well-trained cyclists (time trialists) were recruited from local cycling clubs. These were the same athletes that were recruited in study 2. Inclusion criteria as described in section 3.1 were met. An exclusion criterion was set at 10 mile time-trial time-to-finish of ≥ 25 min in the past season. The mean (± SD) physical characteristics of the participants are given in table 5.1.

6.2.2 Experimental design

The study was designed as a randomised, single blind, placebo controlled crossover intervention. Participants were initially assigned to a four week calcium intervention (1000 mg elemental calcium (citrate)/day). And the following year, four out of the ten participants returned to perform the study without any intervention. The study was designed as a single blind intervention to ensure the collection of the intervention data was not compromised due to the long waiting period between the two points in data collection. Participants were not informed of the single blind nature of the study and were led to believe that the intervention was double blind and they were randomly assigned to either a calcium or placebo group.

The study was repeated at the same time of the year (October to January) to coincide with the end of the racing calendar. This allowed the changes observed in the intervention not to be caused primarily due to increase in training intensity or load that occurs during the racing season. In addition the October to January period is generally used to as a low training period by most competitive cyclists (Sassi et al.,

2008, White et al., 1982a); therefore any improvements observed could be attributed to a large extent on the intervention.

As with the earlier interventions the capsule formulation procedure was adhered to as described in section 3.2.7.

6.2.3 Testing protocols

6.2.3a $W_{peak\ (aerobic)}$ test

All participants underwent a $W_{peak\ (aerobic)}$ test one at a time in order to compare their time trial performance power output with their peak power output. This was performed once either before the 25TT or before 10TT depending on the randomisation protocol. Participants reported to the laboratory in a post prandial state of at least one meal consumed about 60 to 90 min prior to testing. After setting up of cycle ergometer and emptying of bladder, the test was commenced and conducted as described in section 3.3.4.

6.2.3b Time trials

Participants performed two 10 TT, one at baseline before calcium supplementation (10TTC1) and one at the end of the 30 day intervention period (10TTC2). On each occasion the participants arrived post prandial after consumption of the same meal at least 60-90 min prior to testing. Upon arrival they underwent the anthropometry measurements as described in section 3.2.5 and resting blood measurements as described in section 3.2.4 for analysis of lactate, plasma glucose and NEFA. The

tests were performed as described in described in section 3.3.5. Water was allowed *ad-libitum* throughout the tests. No verbal or other forms of encouragement were given during the tests.

At the same time of the following year participants (n = 4) repeated the same study without a supplement intervention (nocebo). They performed a baseline test before the start of the intervention (10TTP1) and after 30 days (10TTP2).

6.2.3c Dietary and physical activity records

All participants were asked to keep a seven day diet diary and physical activity log as described in section 3.2.6 during any typical week of the intervention and were asked to repeat the same in the following year during the nocebo intervention.

A schematic representation of the test protocol has been presented in Fig. 6.1.

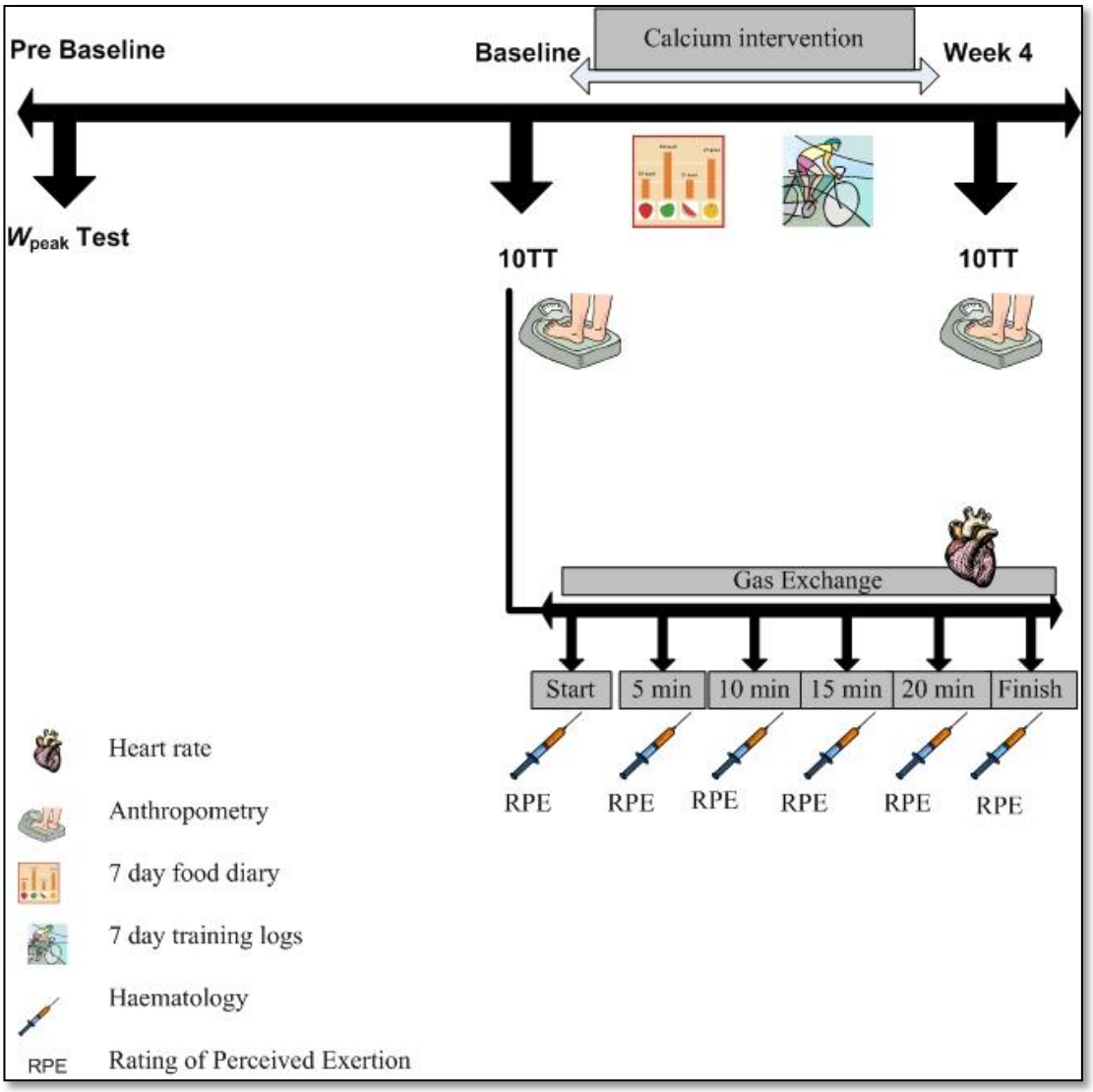


Fig. 6.1 Schematic representation of experimental protocol within each intervention of study 3

6.2.3d Statistical analysis

All data was analysed and statistical analysis conducted as described in section 3.5. Mean differences were analysed for intra-trial changes and not inter-trial changes. This was done to measure the changes due to calcium supplementation from baseline and after four weeks.

6.3 Results

6.3.1 *Performance variables*

Mean changes in performance variables between the two trials before and after calcium supplementation have been presented in table 6.1 along with % change, 90%CI and effect size (*d*). All relevant variables are discussed individually as a product of the time line during the exercise period in the following sections.

Table 6.1 Mean (± SD) change in performance variables in participants of study 3, following calcium supplementation for four weeks

n = 10	10TT C1 (mean ± SD)	10TT C2 (mean ± SD)	% change (mean)	90%CI of mean change	*d*
Time to finish (min)	23.66 ± 1.18	23.45 ± 1.07	-0.86	-0.26 to -0.14	0.18
Power output (W)	264.32 ± 32.56	271.54 ± 33.20	2.66*	6.88 to 7.56	0.21
Relative PO (W/kg)	3.60 ± 0.32	3.69 ± 0.39	2.44	0.05 to 0.13	0.25
Relative PO (W/%FFM)	3.11 ± 0.44	3.17 ± 0.44	1.89	0.00	0.14
Gross efficiency (%)	18.59 ± 0.87	19.78 ± 1.27	1.19†	0.98 to 1.4	1.09
Gross economy (W/l)	64.84 ± 3.00	68.99 ± 4.45	6.01†	3.42 to 4.88	0.82
Energy expenditure (kJ)	2078 ± 231	1928 ± 250	-7.78	-160 to 140	0.19
Heart rate (bpm)	168 ± 10	168 ± 14	0.24	-1.34 to 2.16	0.00
Cadence (rpm)	94.42 ± 8.89	94.45 ± 5.88	0.03	-1.54 to 1.6	0.00

*** significant at p < 0.05 † significant at p < 0.001**

Mean changes in performance variables of the athletes (n = 4) who repeated the trial without any calcium supplementation a year later are presented in table 6.2. These are subsequently discussed individually with the calcium trial variables for comparative analysis.

Table 6.2 **Mean (± SD) change in performance variables in participants of study 3, following nocebo supplementation for four weeks**

n = 4	10TT P1 (mean ± SD)	10TT P2 (mean ± SD)	% change (mean)	90%CI of mean change	*d*
Time to finish (min)	24.27 ± 2.04	24.28 ± 2.11	0.01	-0.05 to 0.05	0.00
Power output (W)	266.13 ± 54.91	261.68 ± 53.97	-1.70	-5.23 to -3.69	-0.08
Energy expenditure (kJ)	1795 ± 426	2087 ± 229	13.99	129 to 454	0.85
Gross efficiency (%)	20.21 ± 0.12	19.10 ± 0.50	-1.11	-1.42 to -0.8	-0.90
Gross economy (W/l)	70.49 ± 0.42	66.61 ± 1.75	-5.82	-4.97 to -2.79	-0.90
Heart rate (bpm)	163 ± 10	168 ± 5	3.06	0.89 to 9.11	-0.65
Cadence (rpm)	92.50 ± 5.51	93.85 ± 2.55	1.44	-1.08 to 3.78	-0.31

6.3.1a Time to finish

There was a non-significant decrease in mean time to finish the 10 mile time trial after four weeks of calcium supplementation (-0.20 ± 0.11 min, p =0.08). The maximum decrease in finish time was -0.93 min and the minimum was an increase of 0.66 min. Individual time to finish after before and after calcium supplementation are presented in Fig. 6.2. Time to finish correlated significantly with PPO in both before (r = -0.91, p = 0.001) and after supplementation (r = -0.90, p = 0.001).

There was no significant difference in time to finish after the nocebo trial (0.00 ± 0.06 min, p > 0.05). The time-to-completion of all participants who completed both the calcium and the placebo trial are presented in Fig. 6.2 (second graph) where all (n = 4) participants performed better in the calcium trial except one who's time-to-completion increased after calcium supplementation (0.66 min).

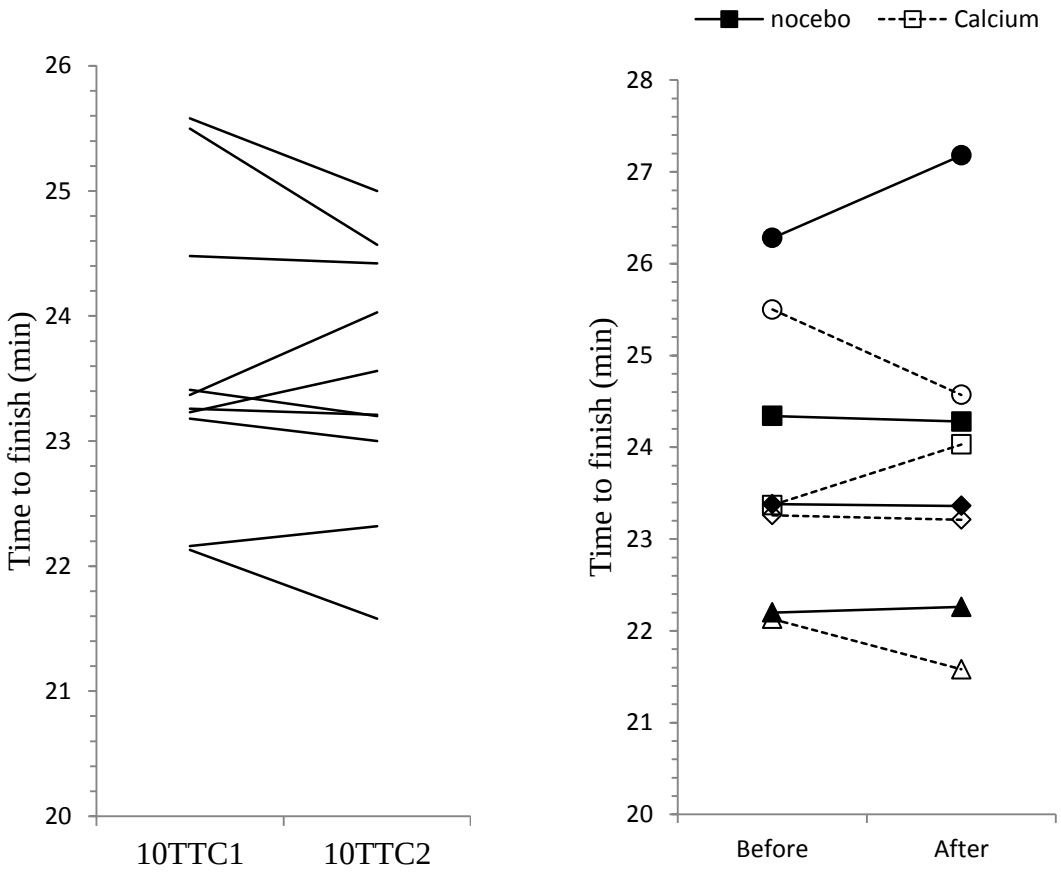


Fig. 6.2 Individual time to finish during the ten mile time trials before (10TTC1) and after (10TTC2) calcium supplementation (n = 10). Time to finish of the individual participants in the nocebo trial before (25TTP1) and after four weeks (25TTP2) (n = 4)

6.3.1b Power output

Mean power output during 10TTC1 and 10TTC2 are presented in Fig. 6.3. After calcium supplementation mean power output was greater throughout the 10 TT ($p = 0.09$). Mean power output in all the trials was greatest in the first five minutes and then steadily declined over 20 min ($p = 0.10$).

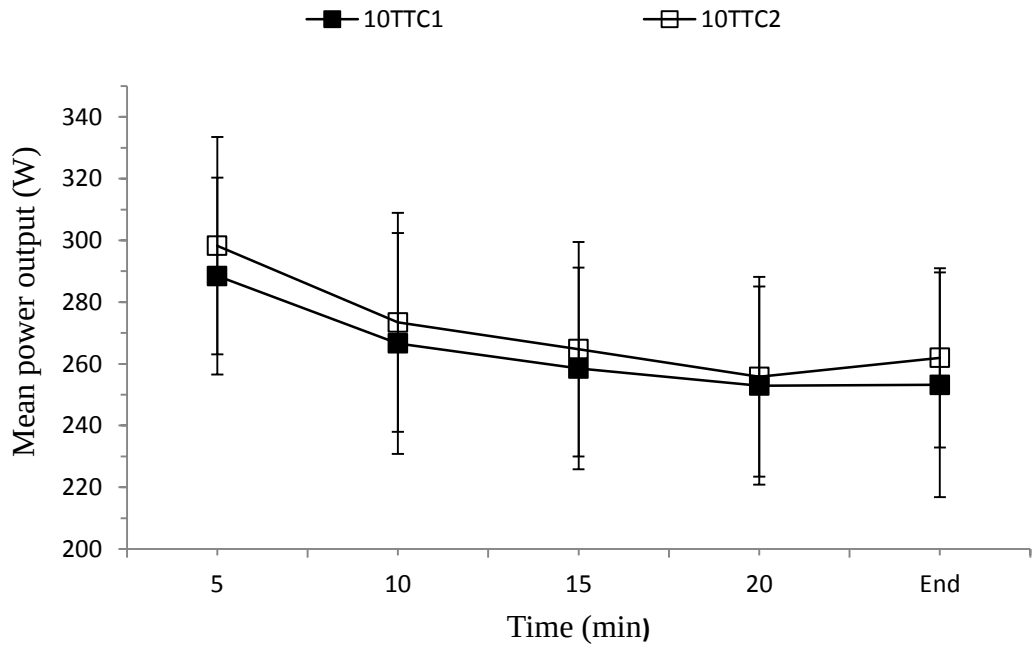


Fig. 6.3 Mean (± SD) Power output during the ten mile time trials before (10TTC1) and after (10TTC2) calcium supplementation (n = 10)

Mean power output in the nocebo trials are presented in Fig. 6.4. In both the trials mean power output was highest in the beginning and steadily declined over the exercise period. Mean power output was lower throughout the exercise session in the 10TTP2 trial ($p = 0.22$). Mean power output was maintained (a mean decrease of 0.43 ± 4.29 W) in the last few min (4.27 ± 2.04 min) in the 10TTP1 trial and mean power output continued to decline in the last few min (4.28 ± 2.10 min) in 10TTP2 trial (-8.12 ± 2.01 W) ($p = 0.27$).

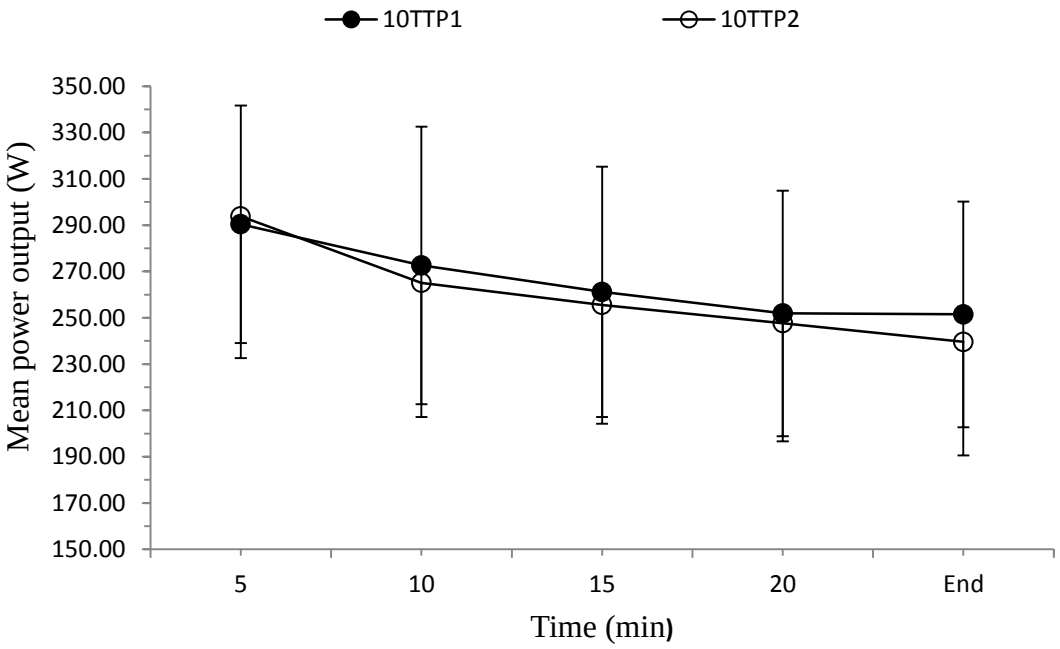


Fig. 6.4 Mean (± SD) power output during the ten mile time trials in the nocebo trial before (10TTP1) and after (10TTP2) four weeks (n = 4)

6.3.1c Gas exchange

i. Oxygen uptake

There was no statistically significant difference in the mean oxygen consumption between the two trials (Fig. 6.5) ($p = 0.11$). There was a significant effect of time in mean oxygen uptake between the start values and all the exercise values ($p = 0.03$).

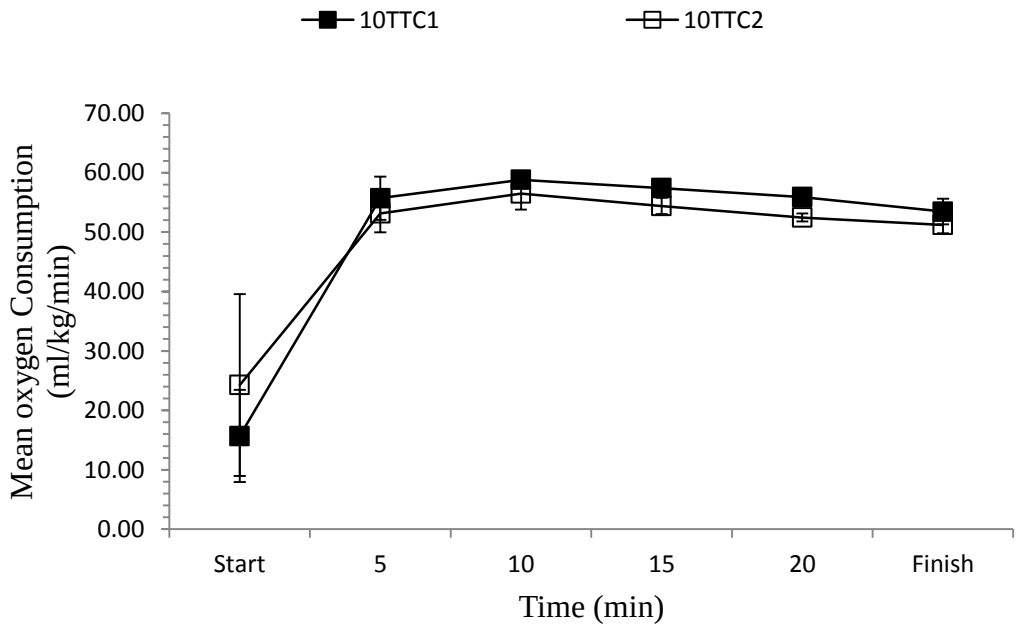


Fig. 6.5 Mean (± SD) oxygen consumption during the ten mile time trials before (10TTC1) and after (10TTC2) calcium supplementation (n = 10)

During the nocebo trial this trend was reversed (Fig. 6.6). The mean oxygen consumption increased during 10TTP2 ($p = 0.12$) and there was no difference in the values at the start of both the trials. There was a significant effect of time between the start and the exercise values in both the trials ($p = 0.02$).

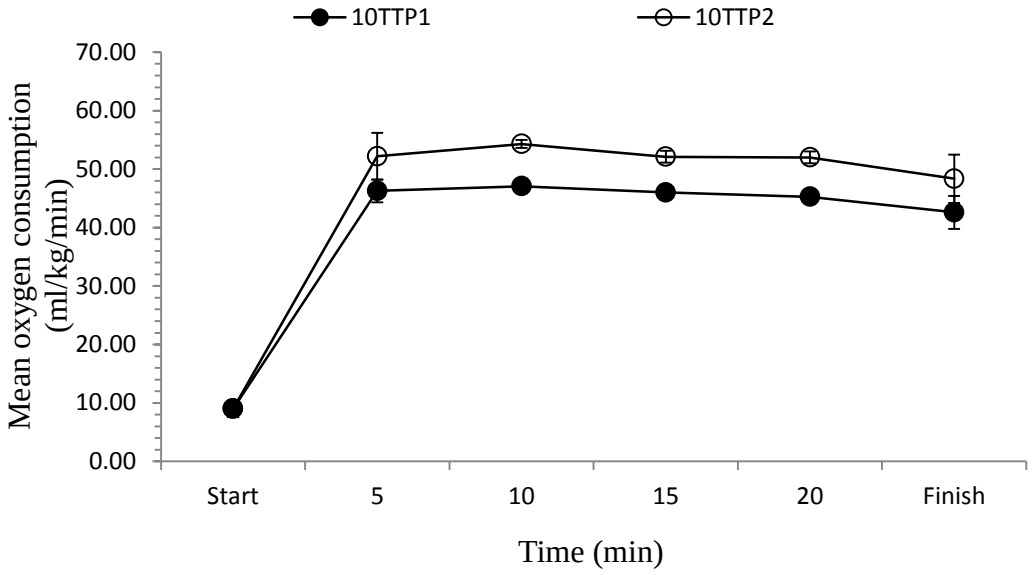


Fig. 6.6 Mean (± SD) oxygen consumption output during the ten mile time trials in the nocebo trial before (10TTP1) and after (10TTP2) four weeks (n = 4)

ii. Respiratory Exchange Ratio (RER)

RER at the start and during trials before and after calcium supplementation is presented in Fig. 6.7. RER increased in the trial after four weeks of calcium supplementation as compared to the trial before, but this did not reach statistical significance ($p = 0.08$). There was a significant effect of time from resting values compared to the exercise values in both trials ($p = 0.03$).

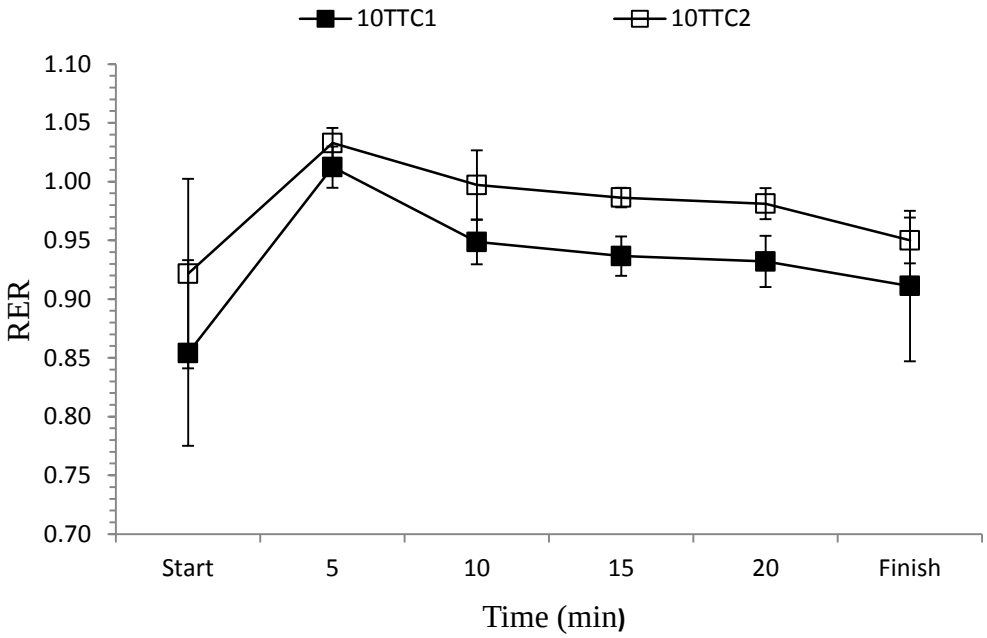


Fig. 6.7 Mean (± SD) RER during the ten mile time trials before (10TTC1) and after (10TTC2) calcium supplementation (n = 10)

This trend was not observed in the trials after one year without calcium supplementation (Fig. 6.8) ($p = 0.28$).

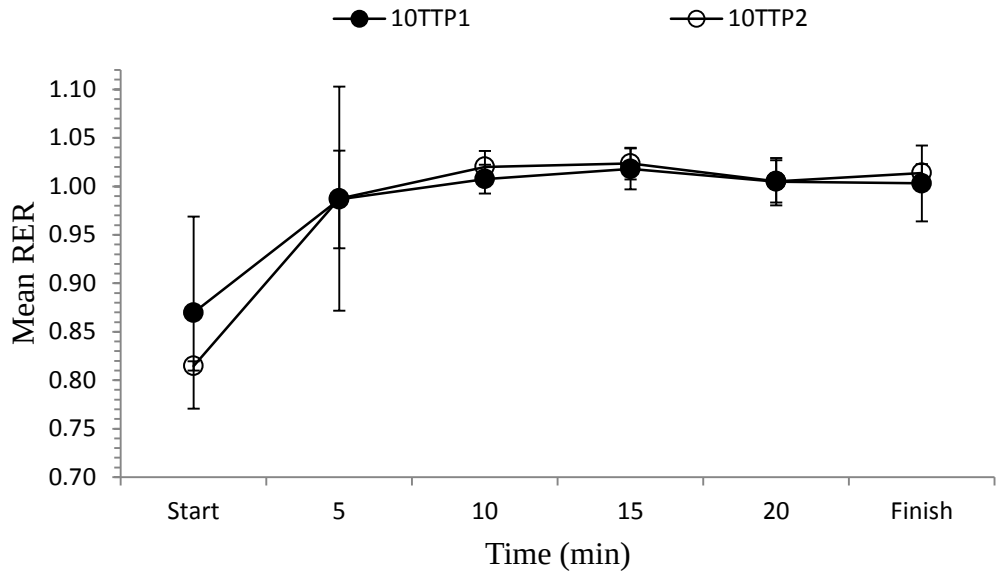


Fig. 6.8 Mean (± SD) RER output during the ten mile time trials in the nocebo trial before (10TTP1) and after (10TTP2) four weeks (n = 4)

6.3.1d Gross efficiency

Athletes produced higher power at a given rate of oxygen consumption after calcium supplementation ($p = 0.001$). This trend was observed across the range of oxygen consumption values during the ten mile time trials. Gross efficiency was significantly higher throughout the exercise session after the calcium supplementation throughout the trial (Fig. 6.9) ($p = 0.001$) compared to baseline.

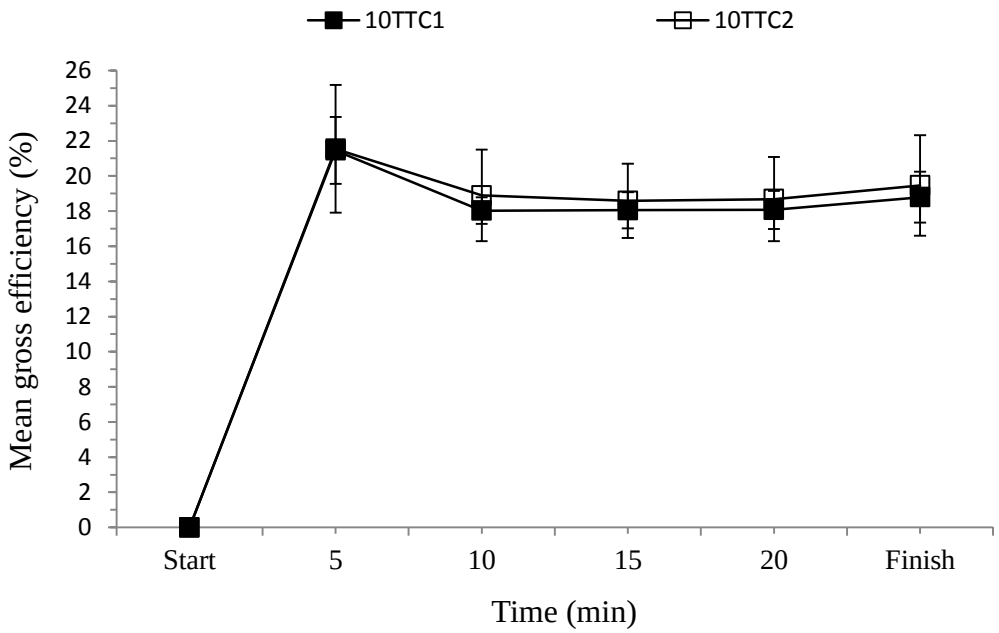


Fig. 6.9 Mean (± SD) gross efficiency during the ten mile time trials before (10TTC1) and after (10TTC2) calcium supplementation (n = 10)

However, this trend was reversed in 10TTP (Fig. 6.10), but did not reach statistical significance ($p = 0.09$).

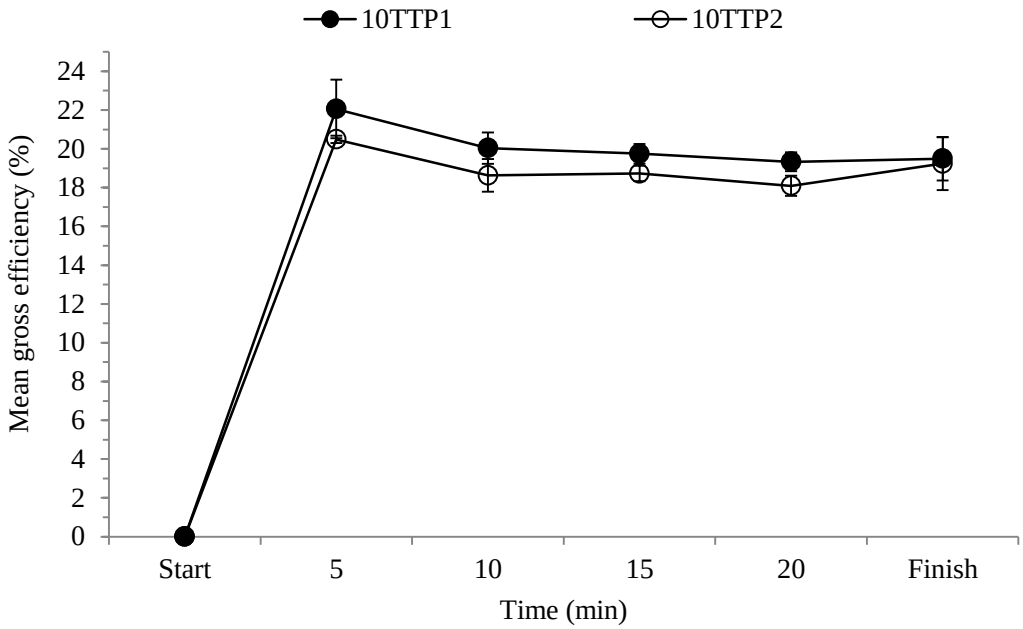


Fig. 6.10 Mean (± SD) gross efficiency output during the ten mile time trials in the nocebo trial before (10TTP1) and after (10TTP2) four weeks (n = 4)

6.3.1e. Haematological variables

i. Plasma lactate

Mean plasma lactate in 10TTC1 and 10TTC2 are presented in the Fig. 6.11. There was no difference in the plasma lactate values at rest between the two trials ($p = 0.18$).

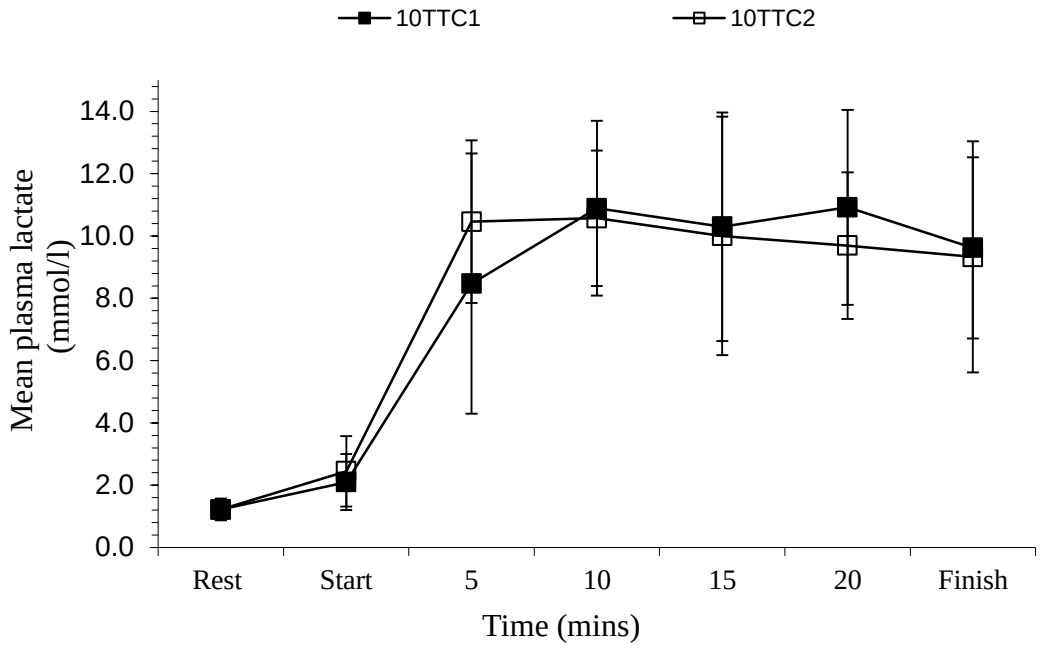


Fig. 6.11 Mean (± SD) plasma lactate during the ten mile time trials before (10TTC1) and after (10TTC2) calcium supplementation (n = 10)

ii. Plasma glucose

There was no statistically significant difference in the mean plasma glucose between the two trials (p = 0.10) (Fig. 6.12). Mean exercise plasma glucose decreased in the first five min in both trials but remained higher in the 10TTC2 throughout the rest of the time trial. There was no statistically significant difference (condition and time) in the values between the two trials but the trend indicated that exercise plasma glucose was higher throughout 10TTP2.

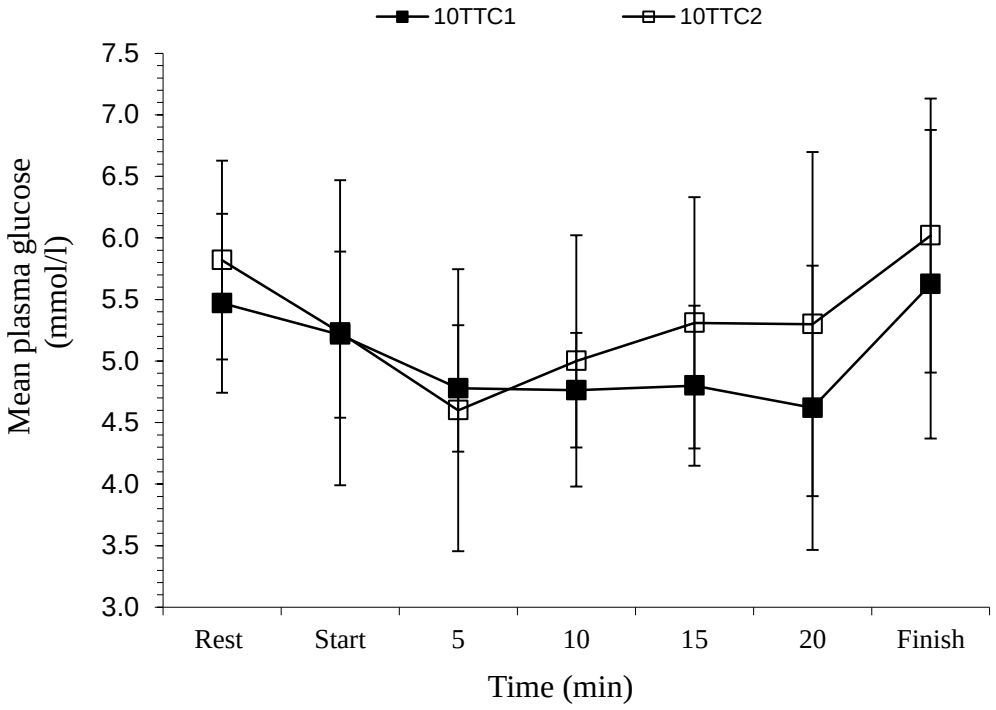


Fig. 6.12 Mean (± SD) plasma glucose during the ten mile time trials before (10TTC1) and after (10TTC2) calcium supplementation (n = 10)

iii. Plasma NEFA

Mean plasma NEFA was non-significantly higher at rest and during exercise following four weeks of calcium supplementation as compared to before supplementation ($p = 0.07$) (Fig. 6.13). Resting values were significantly lower than the NEFA values towards the end of the exercise period (time points 20 min and finish time) in both the trials ($p = 0.01$, main effect of time).

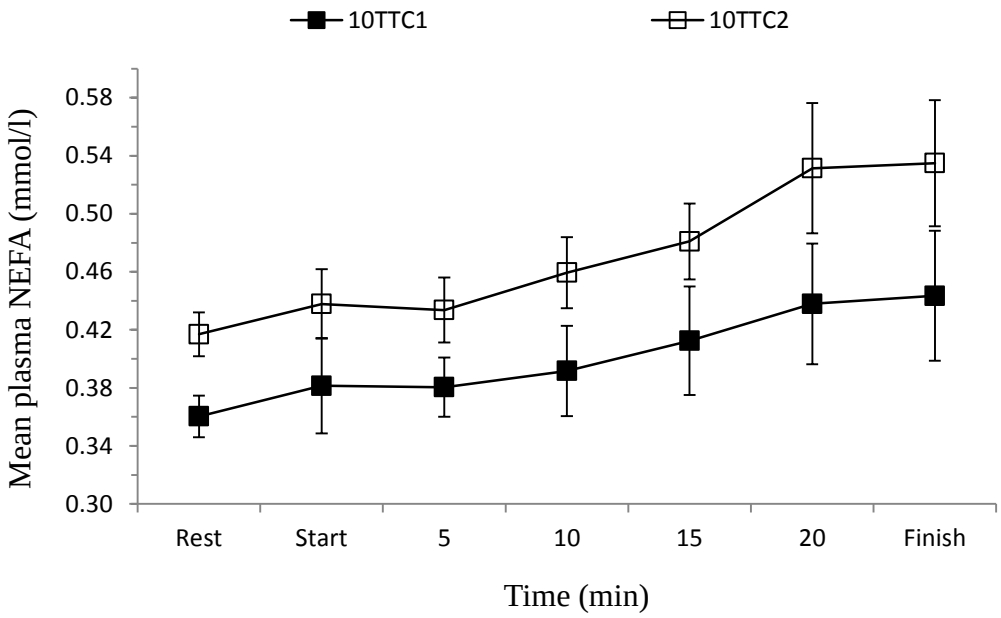


Fig. 6.13 Mean (± SD) plasma NEFA during the ten mile time trials before (10TTC1) and after (10TTC2) calcium supplementation (n = 10)

6.4 Discussion

6.4.1 Performance enhancement via attenuation of muscular fatigue

The principle aim of this study was to continue to quantify the effect of calcium supplementation on cycling performance. This study aimed to outline the effects of the supplementation on a more intense and relatively higher contribution of the anaerobic fraction of energy supply (~75-80% PPO) during exercise. The main findings indicate that calcium seems to improve cycling performance even at a higher intensity but the magnitude of benefit seems to diminish. The cyclists in this study (10 mile time trial) were 0.86% faster to finish after four weeks of calcium supplementation than baseline measurements. Time to finish the trial in this study correlated significantly with the PPO of the riders in both trials, baseline and after supplementation and this concurs with Hawley and Noakes (1992) who looked at PPO and 20 km TT correlations (r = -0.91) but not Balmer et al. (2000) who reported high correlations with PPO and mean PO of 16.1 Km TT (r = 0.99) but not with time to finish (r = 0.46). This result is not as clinically significant as the performance enhancement achieved in the 25 TT (change in time to finish = 2.25%).

Bentley et al. (2001) have shown a strong correlation on a 90 min TT performance with W_{peak} but not that of 20 min TT. Even though these relationships have been criticised in the literature (Bentley et al., 2001) for differences in the protocols used to elicit W_{peak}, it does indicate that the mechanisms that are involved in improvement of submaximal exercise due to increased dietary intake of calcium also play a part at higher intensities. Increased mean PO (7.22 ± 0.65 W) in this study lends to the explanation, that the fatigue attenuating mechanisms have a strong role to play in the

effect that calcium seems to exert as an ergogenic aid. It has also led to a higher GE and economy in the riders and as discussed earlier, Coyle (1992) has eluded towards GE being a key determinant of endurance performance. Once again the riders were riding at a higher given power output with a decreased oxygen consumption level for that intensity after four weeks of calcium supplementation compared to the baseline measurements. GE values in other 10 mile (16.1 km) TT are in the similar range (Balmer et al., 2000).

Coyle (1999) highlights that performance $\dot{V}O_2$ is determined mainly via maximal oxygen uptake and the corresponding oxygen consumption at lactate threshold. The model further indicates that lactate threshold power is a function of lactate threshold $\dot{V}O_2$ and GE. Indeed this study shows that power output was higher in the trial following calcium supplementation at lactate threshold (mean increase of 3.11 ± 0.33%). However this increase in sustainability of increased power output at the lactate threshold was not a function of increased $\dot{V}O_2$, therefore one has to assume that the relationship could have been uncoupled due to the influence of calcium, perhaps in lactate clearance or in the total ionic shift per se from intra to extracellular space. The evidence to support this explanation can be found in the difference in the pattern of appearance of lactate in the blood between the two trials. The 10TT calcium intervention did not see an increase in lactate values above that noted at baseline after the first 5 min of exercise whereas, in the 25TT the mean plasma lactate values were higher than baseline throughout the exercise period. In contrast to the 25TT where the lactate and the power output values followed the same pattern, this was not the case in the short, more intense 10TT despite similar increases in power output after calcium supplementation. The increase in dietary calcium causing a change in the intracellular environment of the muscle could have made it possible

for a higher lactate level to be sustained in the muscle cells upon increased power output before an increase in blood lactate levels which were observed during the 20 min and beyond measurements in the submaximal tests. Therefore it is possible to see the mean lactate values in the blood to increase above that observed during baseline measurements in the 25 TT but not in the short duration ~20 min of 10TT where the muscles could still sustain the higher levels of lactate build up. Studies by Wasserman et al. (1986) show that mechanisms for lactate increase in blood during exercise is inconsistent with mechanisms depending simply on an increase in glycolysis without an accompanying change in the redox state. Bentley et al. (2001) have calculated that only 45% of the variation in the power output during a 20 mile TT could be explained by the relationship between power outputs at lactate threshold. This seems to indicate that other factors aside from the ability of the athletes to delay lactate accumulation in blood may explain the difference in the time trial performance of the two trials.

Various studies looking at the skeletal muscle damage have alluded to the increases in resting $_{i}[Ca^{2+}]$ levels in fatigued muscles compared to the un-fatigued muscle fibres (Bruton et al., 1998, Cairns and Lindinger, 2008, Chin et al., 1997, Westerblad and Allen, 1996). A pivotal role for $_{i}[Ca^{2+}]$ in modifying ec-coupling was first suggested by the finding that in skinned fibres exposed to millimolar concentrations of calcium, ec-coupling was completely abolished (Fitts and Balog, 1996). This in conjunction with the results from other studies (Zhao et al., 2005, Germinario et al., 2008) showing increased availability of extracellular calcium entry pathways into the cells, attenuate fatigue and lowers extracellular calcium exacerbates fatigue during repeated tetani may imply a role of increased calcium intake in these fatigue attenuating mechanisms. The exact mechanism however seems seeped in limitations

of measurements, an in-depth understanding of ionic shifts during high intensity and submaximal exercise and the application of different exercise protocols to study fatigue and performance. An intriguing question, which has been addressed in many studies, is whether cycling efficiency can be increased either by training (Faria et al., 2005, Hawley and Stepto, 2001, Joyner and Coyle, 2008) or any other mechanisms. If calcium did play a part in improvement of GE in the 10 TT then the involvement of cardiac output and pedalling rate were not major players as shown in this trial. Interestingly, Gaesser and Brooks (1975) have implicated pedalling frequency as an important determinant of GE. In this study, it has been shown that an increase in PO with a concurrent reduced oxygen uptake, allowing the athletes to maintain this higher intensity this was achieved with no difference in the pedalling rate or change in oxygen cost. One of the reasons for a decreased oxygen consumption and thus improved GE could be attenuation of fatigue via changes in metabolic costs to sustain the exercise including but not limited to changes glucose metabolism.

6.4.2 Substrate metabolism

During the 10TT glucose concentration in the plasma increased after calcium supplementation after the first five min of exercise compared to the baseline measurements. Studies looking at the relationship of dietary calcium to glycolysis indicate the role of increased $_{i}[Ca^{2+}]$ and a corresponding increase in glucose transport in the skeletal muscle cells (Sørensen et al., 1980). Persechini and Cronk, (1999) demonstrated that in skeletal muscle fibres entry of external calcium could activate calmodulin associated in the mobilisation of hexokinase and phosphofructokinase. Calmodulin activation also increases translocation of GLUT-4

containing vesicles to the sarcolemma (Bruton et al., 2001) subsequently increasing glucose transport in the cells. High intensity (70-80% $\dot{V}O_{2max}$) exercise stimulates glycolysis (Romijn et al., 1993) and it is possible that increase in dietary calcium reducing the $_i[Ca^{2+}]$ rise may reduce or inhibit the glucose uptake facilitated by the activation of calmodulin. This mechanism has been shown to work in insulin stimulated glucose transport into the sarcolemma (Bruton et al., 2001) as well. This in turn could lead to a decreased uptake of glucose in the muscle cells and a potential rise in plasma glucose due to increased glycolytic activity during high intensity exercise. There is also some evidence that growth hormone increases $_i[Ca^{2+}]$ and blocks the blocks the insulin like response of cells in the uptake of glucose (Gaur et al., 1998), however the mechanisms still remain unclear. For example the correlation between exercise induced GH release and its effect on $_i[Ca^{2+}]$ levels in skeletal muscles and whether these influence insulin sensitivity during exercise remains unclear. Therefore even though GH has been shown to affect $_i[Ca^{2+}]$ levels (Schwartz et al., 1992) , its role in glucose metabolism during exercise is unclear. Since high intensity exercise stimulates GH and consequently increases blood glucose levels via increased liver output (Pruett, 1971), if increased dietary calcium did influence GH this could be an important mechanistic pathway for the increased blood glucose levels seen in the trial after four weeks of calcium supplementation. Also, increased adrenergic stimulation of glycolytic activity simply as a function of increased intensity of exercise cannot be ruled out. This can be seen even in the increase of NEFA in the blood after calcium supplementation compared to baseline measurements. Lipolytic activity seems to have been enhanced due to increase in calcium intake. This is evident from both the 10TT and 25 TT data and there is evidence in the literature to suggest that increase in metabolites in the plasma may be

due to the role calcium plays in energy and substrate metabolism in addition to its role in attenuating fatigue and thus enabling the athletes to maintain higher power outputs.

6.4.3 Other possible avenues in performance enhancement via calcium

Blood flow depends on vascular tone, which is greatly influenced by the sympathetic nervous system through adrenoceptor stimulation and ultimately by the intracellular free calcium (Kuriyama et al., 1982). Jolma et al. (2000) have shown that increased dietary calcium in hypertensive people reduced vasoconstriction via increased sensitivity to nitric oxide and decreased vascular production of superoxide and vasoconstrictor prostanoids. The participants in this study maintained a higher power output without a concurrent increase in oxygen consumption, which allowed them to be more efficient after four weeks of calcium supplementation. It is possible that improved vasodilatation due to increased dietary calcium, led to improved blood transport and thus oxygen to the muscle cells, thus improving GE.

6.4.4 Conclusions

The findings from this study further support the role of calcium in endurance performance. As with the previous study the more intense 10TT also elicited higher PO and concurrent faster time to completion. In addition the similar trend of increased GE of the riders was seen as in the longer duration event of 25TT (thus supporting hypothesis v). As suggested by the literature (Romijn et al., 1993, Horowitz and Klein, 2000) the 10TT relied mainly on non lipid substrates for energy

production. The appearance of FFA continued to rise throughout the exercise however the RER data suggests that fat metabolism played a minor role in the performance of this event (thus supporting hypothesis vi).

Thus despite the role of fat metabolism being limited in an event of 10TT, calcium positively influenced performance of the riders. This indicates that beneficial role of calcium in performance enhancement is broader than just its effects on substrate metabolism.

CHAPTER 7

SYNTHESIS OF FINDINGS

This thesis set out to investigate the role of supplemental calcium in substrate metabolism, body composition and thus endurance performance. In order to study these effects, a series of investigations were designed. First, was to explore the changes in substrate metabolism, particularly increased fat oxidation during exercise. These changes were then cumulated along with the effect of calcium supplementation on body composition changes over four weeks. Lastly, based on the findings of this exploratory study, sample population to study the effects on calcium directly on performance enhancement was identified and studied.

The first intervention of moderate intensity exercise, on a heterogeneous mixed population, was designed to explore changes in substrate metabolism due to calcium supplementation. Based on the suggested *in-vitro* data (Xue et al., 2001, Zemel, 1998, Zemel, 2001) and epidemiological studies (Teegarden et al., 2008, Teegarden and Zemel, 2003, Zemel et al., 2008, Zemel et al., 2000), the initial model derived from the literature (Fig. 2.17) suggests an inverse relationship between low dietary intakes of calcium and decreased lipolysis in intact adipose tissue (Xue et al., 2001), which was also related to changes in iPTH levels. Therefore, the first stage in the

creation of a detailed model, to identify the role of calcium supplementation in sports performance, was to identify if calcium supplementation had an effect on calcium metabolism. Results from the first study revealed that calcium supplementation did not significantly change any markers of calcium metabolism in the non-deficient healthy people. Plasma iPTH did show a small non-significant reduction in the calcium supplementation trial, similar to the findings of several other studies (Feldman, 1999, Schrager, 2005, Theobald, 2005b).

Secondly, it was important to study the impact of calcium supplementation in the diet on markers of lipolysis as suggested by Xue et al. (2001). This may help changes in substrate oxidation rates during endurance type activities. Indicators of lipolysis such as appearance of NEFA in the plasma showed a non-significant increase in the calcium supplementation trial, along with increased fat oxidation during the exercise session. Even though the markers of calcium metabolism in the plasma saw no changes due to increased calcium, plasma NEFA did show a trend towards increased lipolytic activity. This is shown within the preliminary model outlined in Fig.7.1.

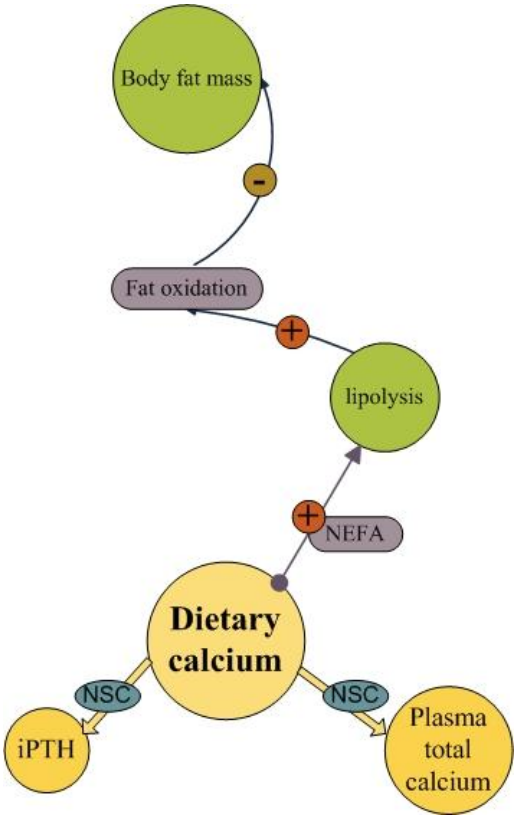
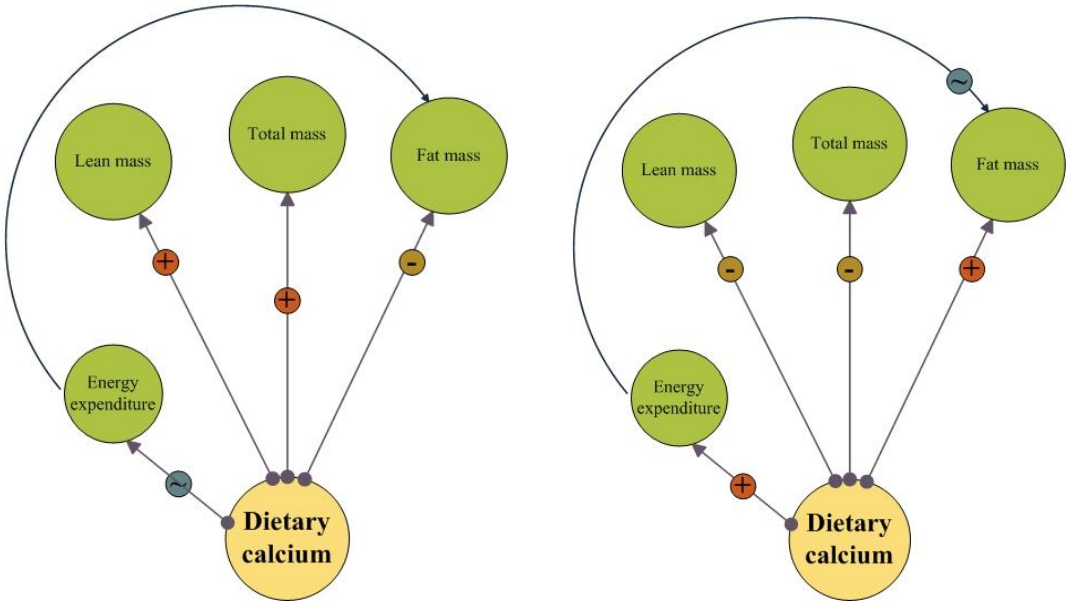


Fig. 7.1 Schematic representation of changes in blood metabolites aftercalciumsupplementation (stage1). Non-significant changes (NSC)

This increased availability of NEFA could potentially increase fat oxidation rates. Fat oxidation at rest has been shown to increase modestly in healthy population as an effect of increasing calcium (Jacobson, et al., 2005) and dairy (Boon et al., 2005) in the diet. This has not been studied in exercising population prior to this investigation. Indeed, the results from the first study, allied to those existing in the literature, showed that fat oxidation rates, at moderate intensities of exercise, were significantly higher after four weeks of calcium supplementation. The second stage of creating a model to explain calcium supplementation and performance consisted of studying the impact of these substrate oxidation changes over a four week period on body composition.

Favourable changes in body composition due to increase in dietary calcium have been reported elsewhere (Pereira et al., 2002, Zemel, 2004, Zemel et al., 2005) in obese, over-weight non-athletic populations. The current study is the first of its kind studying athletic populations. The results from study one showed that the participants lost significant amounts of body fat after calcium supplementation. However, these results, when analysed according to training status, showed that this was the case only with the well-trained participants. The recreationally trained participants displayed increases in body fat, and decreases in lean mass. These findings are expressed within the model at Fig. 7.2.



(a) (b)

Fig. 7.2 Schematic representation of the changes in body composition of well-trained (a) and recreationally trained (b) participants in study 1(stage 2)

The difference in the change in body composition, between the two sub groups, was not related to TEE. Mean TEE in the entire group was significantly higher, though this was not the case when the data was analysed for well-trained athletes (Fig. 4.15). Unlike animal model observations, the well-trained athletic sample did not display the energy inefficiency model described in Zemel et al. (2000). This model alluded to the down regulation of UCP2 and 3 as a result of higher circulating calcitriol levels (Fig. 2.17) during exercise. However, if this were to be true only in regulating RMR as suggested (Sun et al., 2004, Zemel et al., 2000) then the decreases in body fat levels of the well-trained participants, despite no increase in TEE during exercise, could be explained. Irrespective, these changes in body composition could have an indirect effect on performance enhancement in endurance sport, especially in events that require the athletes to maintain a power to weight ratio advantage over a period of time (Coyle et al., 1991).

When further building the model, the changes in body composition, with calcium supplementation, are now embedded at Fig. 7.3 This model consolidates the findings from the initial exploratory study, on where calcium supplementation may play an important role on improved lipolysis and ensuing fat oxidation, and its effects on changes in body fat levels.

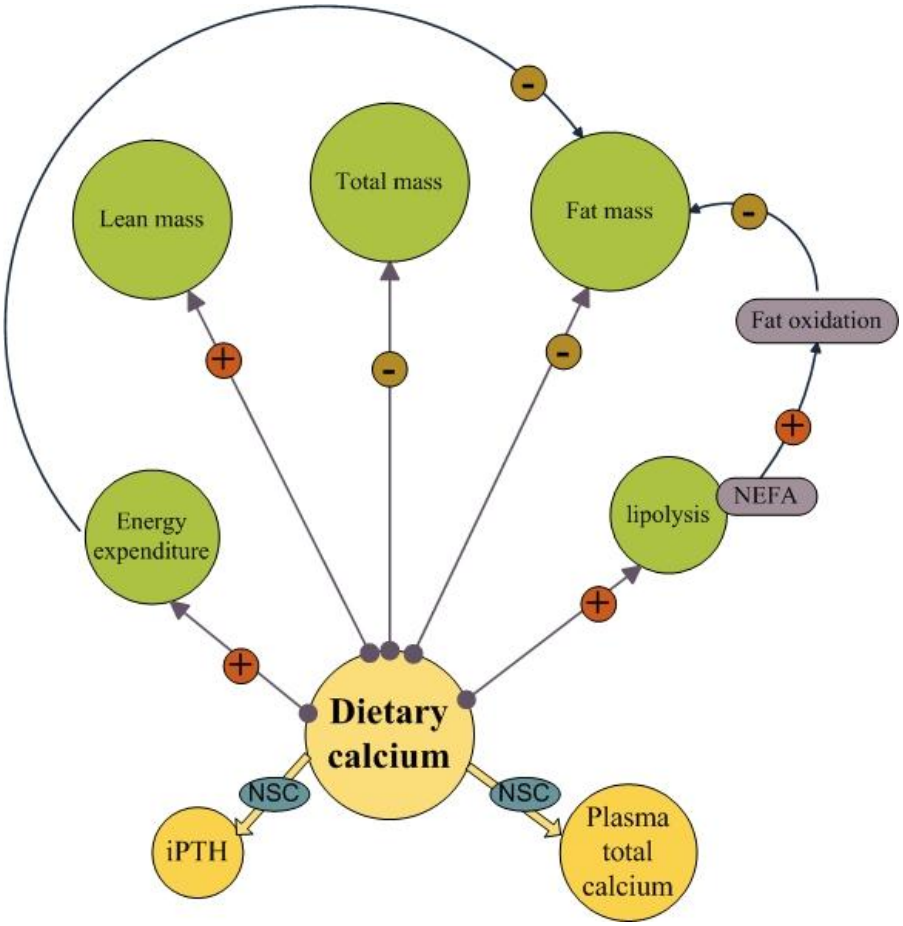


Fig.7.3 Schematic representation (derived from the mean of the entire group in study 1) of the effect of calcium supplementation on fat metabolism and body composition changes in four weeks of supplementation. Non-significant changes (NSC)

The final stage of the model creation required identification of the impact of the changes mentioned above on athletic performance enhancement. In order to identify the direct effect of calcium supplementation on performance, studies two and three were performed on well-trained, time trial cyclists. Stratification of performance, based on preliminary time trial efforts, was used to ensure as homogenous a population as possible were used. Measures of energy and fat metabolism, body composition changes were also measured to corroborate the findings from the previous study, and to further support the structure of the newer model outlined in Fig. 7.3. Body composition changes followed the same trend as in study one, again

justifying their inclusion as a key component within the model. The effect of calcium supplementation on lipolysis too concurred with the earlier study, as seen by the increased availability of NEFA after four weeks of supplementation. These results further consolidated the role of calcium in affecting energy and substrate metabolism in highly trained athletes, and justify their inclusion within the performance-related model.

However, the novel finding in these studies was that this increased availability of NEFA did not influence the rate of carbohydrate oxidation during a time trial. This confirms that fat oxidation rates are tightly regulated as a function of intensity of work (Romijn et al., 1993) which may rise during competition to override the lipolytic drive and increase reliance on glycolysis. Despite calcium supplementation being non-glycogen sparing it aided in decreasing time to finish the trials; both 25TTC and 10TTC. One explanation for this can be seen in the improved gross efficiency of the participants after the calcium intervention. Despite a mean decrease in consumption of oxygen during the time trials the PO increased (~4% increment in power output during the 25 TT and ~2.7% improvement in the power output in 10 TT) decreasing time to finish as a consequence.

The role of calcium in the increased PO in the time trials could be attributed to its effect on attenuating fatigue via increased in extra cellular calcium levels. Cairns and Lindinger, (2008) reported attenuation of muscular fatigue with increased extra cellular calcium levels. Westerblad and Allen (1991) showed that in exercise that lasted longer than 20 min muscles did not display marked acidosis during fatigue but increased i[Ca^{2+}]. Calcium supplementation attenuates increases in i[Ca^{2+}] (Zemel, 2002). It is therefore plausible that the higher intensity of exercise sustained by the athletes, post calcium supplementation, was a result of fatigue attenuation via the

above mentioned mechanisms. This response has been embedded within a further update to the model, seen in figure 7.4. Thus, the increased plasma lactate in the 25TTC2, compared to that in 25TTC1, is potentially a function of increased PO (Fig. 5.16). In addition, the reduction in oxygen uptake which contributed to the improved gross efficiency has potential associations with improved vascular tension due to increased intake of calcium, as highlighted by the research carried out with hypertensive patients (Zemel, 2001, Azadbakht et al., 2005, Pereira et al., 2002, Fujiwara et al., 1991, Stern et al., 1984). This may allow for increased distribution of oxygen to the muscles whilst exercising reducing the gross uptake of oxygen. These influences are now embedded in the revised model at Fig. 7.4.

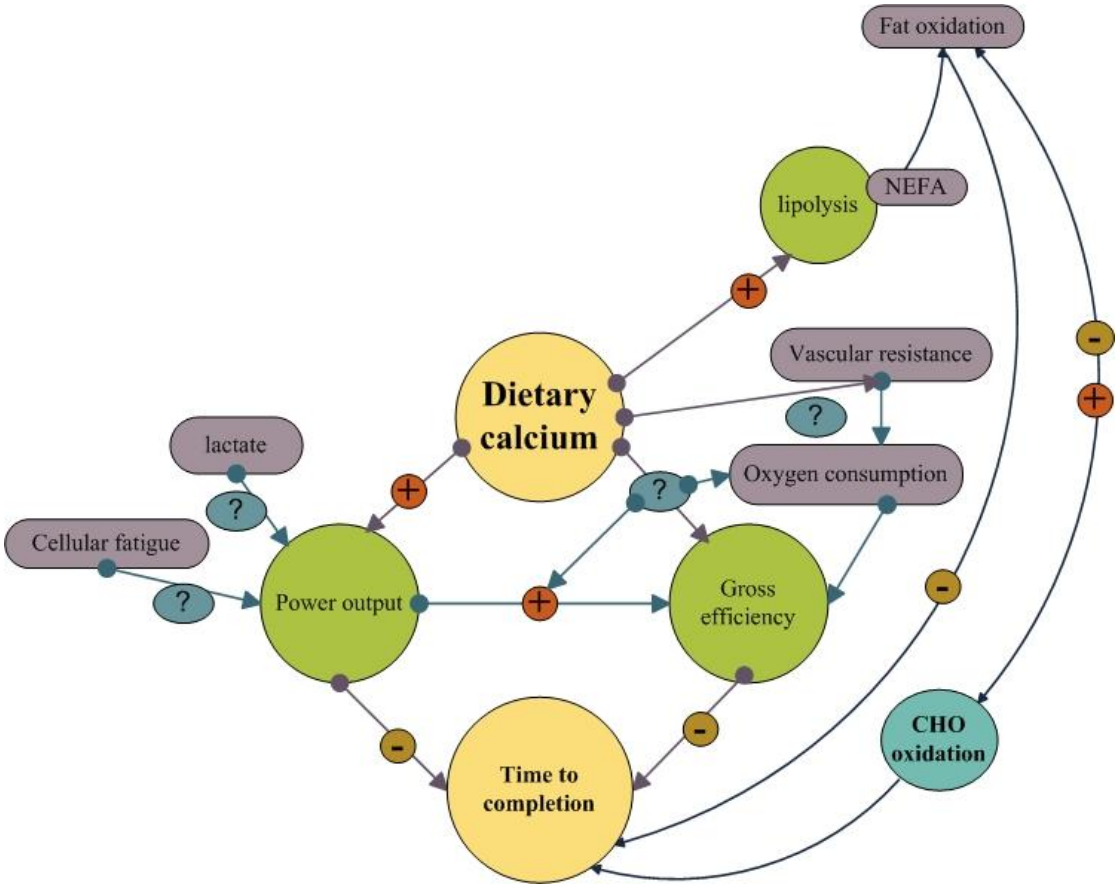


Fig.7.4. Schematic representation of the role of calcium in performance enhancement via its effect on PO, GE

The identification of the possible mechanistic routes in which calcium may have had an influence on performance in addition to its effect on body composition changes demonstrate that calcium supplementation has a dual effect in improving performance. Thus, the final stage in understanding the model, in which calcium may function as an ergogenic aid, is presented in Fig. 7.5. This model incorporates the findings from this series of hierarchical investigations, where the role of fat metabolism and oxidation having been explored in a heterogeneous population, led to performance tests conducted on well-trained athletes. In particular, the role of body composition, performance, hormonal influences and vascular factors are outlined.

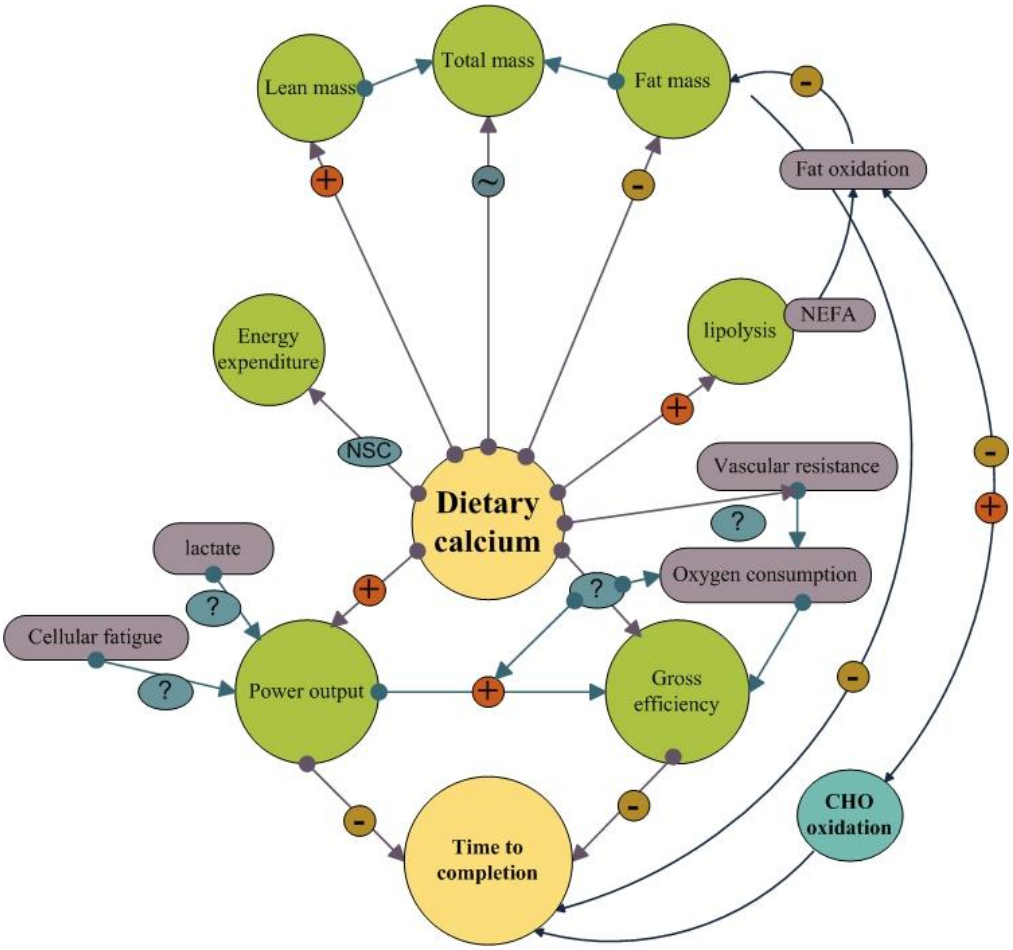


Fig.7.6. Schematic representation of the consolidated role of calcium in performance enhancement via its effect on PO, GE and changes in body composition. Non-significant changes (NSC)

Results from the final studies revealed that time to completion of the endurance trials decreased uniformly. The milieu of possible avenues is described in Fig. 7.5, though further investigation is needed to confirm the existence of these pathways more fully. However, what the completed model does not show was that performance enhancement in endurance exercise may have occurred via glycogen sparing. The role of increased mobilisation of fatty acids into the plasma has an important function in the overall effect it may have on changes on body composition. During a moderate to high intensity performance trial, this increased availability of fatty substrates is still over ridden by the preferential use of carbohydrate sources (Brooks, 1997).

Since all the interventions were four weeks long it has only been possible to study short term effects of these metabolic shifts proposed by Zemel and co-workers. They proposed that calcium's down regulation of calcitriol synthesis is primarily responsible for its beneficial impact on body weight (Zemel et al., 2000). This view is based on *in-vitro* studies in which treatment of adipocytes with supraphysiological (nanomolar) levels of free calcitriol produces a rapid influx of calcium. Such an effect is observed almost immediately and is not mediated by the interaction with the nuclear vitamin D receptor (Shi et al., 2001b). The translation of these *in-vitro* observations to human studies will take time in gathering a critical mass. But more importantly these observations need to be made specifically on athletes per se as physiological adaptations in energy and substrate metabolism are rather different in trained and untrained people (Sidossis et al., 1998). This was highlighted by the difference in the body composition results in the two sub-groups in study 1. Such factors including the lack of statistically significant results in certain metabolic variables suggest that these observations need to be made on a larger sample of

athletes with different training status. In addition, there are a few limitations that were encountered as a process of this investigation which have been highlighted below.

7.1 Limitations of the study

7.1.1 Implications of nocebo interventions

Since there was no placebo treatment in Chapters 5 and 6, the sensitivity of the magnitude of the findings may be compromised. The main reason for not having a cross-over design was to ensure that the intervention period was short enough to not include any change in normal training volume of the athletes due to the racing calendar. The four week period of the intervention during the months of October to January for all the 10 athletes allowed maintanence of this homogeneity. Based on the study one (Chapter 4) logistics, a cross over design incorporating a four week intervention period would transcend this low volume training period. This would lead to a bigger loss of sensitivity of the findings compared to the exclusion of a placebo. In order to minimize the potential negative effects of this single blind test, retest design, two things were ensured; firstly that the athletes were told that the design was a two group intervention where they could either be in the placebo or the intervention group; and secondly, the time trials were conducted again at the same time of the following year with a smaller proportion of the same participants (n = 4) who could participate again and this allowed for reporting the changes in performance that would normally occur at that time of the year when no calcium supplementation was taken. The limitation of this trial in the following year was that the sample size was less than that of the original calcium supplementation trial

(40%) and thus compromising an exact like for like findings. However, a strength of this designed was that participants acted as their own controls, thus enabling a comparison of an individual's data with and without calcium supplementation. Even though, this did not reach statistical significance, meaningful individual differences have been observed in the outcome between the nocebo and the calcium supplementation trials.

7.1.2 Sample size

Another limitation of all the studies has been the limited sample size. It has been reported that a sample size of 10 participants with 3 trials would not meet the 95% confidence to study within participant variations (Hopkins et al., 1999). However, externally valid test protocols such as time trials (Atkinson et al., 1999) have allowed for the demonstration that the changes in performance indicators such as power output and time-to-completion to show meaningful changes in performance of the participants. As discussed by Batterham and Atkinson (2005) the sample size matters only if the statistical significance is not reached. It has been shown that GE and time-to-completion in at least 25 TT and GE and mean power output in 10TT are statistically significant results. In conjunction with the statistical significance and the 90% CI data one can confidently say that the sample size was adequate to reduce the probability of a type I error. Batterham and Atkinson (2005) argues that a low sample size makes it more difficult to arrive at statistical significance because the associated low degrees of freedom make it more difficult for a given test statistic to be greater than the critical value. Lastly, sample sizes in sports science studies have traditionally been smaller in number compared to other medical and clinical trials.

One reason for this is the homogeneity of the sample required to test the effect of a certain intervention. This invariably will reduce the transferability of the results to a wider population base and therefore any conclusive evidence of the ergogenicity of calcium in the wider sporting areas needs further research.

7.1.3 Normal variance of cycling performance

One disadvantage of the studies on 25 and 10 mile TT was that the coefficient of variance of the cyclists' performance on time trials was not measured without any intervention in general. (Hopkins et al., 2001) suggests the importance of a practice trial as more evident in studies conducting more than two trials. (Hopkins et al., 2001) have reported the CV between the first 2 trials as 1.3 times the CV between subsequent trials; performance was also reported to have improved by 1.2% between the first 2 trials but by only 0.2% between subsequent trials. However, they found no clear-cut effect of time between trials (Hopkins et al., 2001). All the performance variables (time-to-completion and mean PO) however have improved by more than 1.2% with the exception of time to finish in the 10TT (-0.86%) and the trend being reversed in the trial that followed at the same time after one year without any intervention indicates that the ergogenic effects seen in the trials as a consequence of the calcium intervention is not a type I error.

7.1.4 Ergogenic effects of citrate

The form of calcium supplements used in all the studies was calcium citrate. This was chosen as calcium citrate has been shown to have improved bioavailability in humans compared to other oral forms (Hanzlik et al., 2005, Heaney et al., 2001, Sheikh et al., 1987, Pak et al., 1987). However, citrate supplementation has been studied in sports as a potential ergogenic aid (Oöpik et al., 2003, Potteiger et al., 1996, Kowalchuk et al., 1989, Parry-Billings and MacLaren, 1986). One of the main biochemical arguments in the case of the ergogenicity of citrate is the increase in extracellular pH which may create a favourable pH gradient for the removal of intracellular lactate and hydrogen ions (Requena et al., 2005). In most studies this was achieved via an acute load of calcium citrate given 30 mins (Hooker et al., 1987) ranging to 3 hours (George and MacLaren, 1988) prior to the exercise session. A majority of the positive outcomes reported have been with studies looking at high intensity, near maximal (Mc Naughton and Cedaro, 1992) to supra maximal (McNaughton, 1990, Linossier et al., 1997) with a few studies reporting some beneficial effect of citrate in endurance events (Potteiger et al., 1996, Oöpik et al., 2003) but not others (Schabort et al., 2000). In order to minimize any effect of citrate on performance the participants were asked to consume their capsules at least 60 to 90 min prior to their training sessions during the four weeks of calcium supplementation. The laboratory tests in all the studies were performed after the end of the supplementation period, so no capsule was consumed on those days. There are no known interventions looking at any longitudinal effects of citrate on performance since citrate associated buffering capacity does not lend to these effects on long term accumulation of these ions.

7.1.5 Other physiological variables

In terms of physiological parameters studied, there are a few shortcomings in variables measured the author would like to highlight. It would have been very interesting to study the effects of changes in resting metabolic rate and core body temperature measurements of the participants due to calcium supplementation. It has been shown that increasing dietary calcium increases lipolysis (Xue et al., 1998, Xue et al., 2001) and decreases lipogenesis (Xue and Zemel, 2000). Also UCP 2 in adipocytes and UCP 3 expression in skeletal muscle have been shown to be significantly up regulated in high calcium diet interventions (Sun and Zemel, 2004). These proteins are able to uncouple ATP production from mitochondrial respiration, thereby dissipating energy as heat and affecting energy metabolism efficiency (Schrauwen and Hesselink, 2002). Thus, the changes observed in body composition in all the studies in this trial may have had a stronger explanation and perhaps a direct mechanistic link if resting metabolic rate and core body temperature observations were made.

CHAPTER 8

CONCLUSIONS AND FUTURE DIRECTIONS

8.1 Overall conclusions

The aims of this investigation were to explore the *in-vitro* mechanisms of the role of calcium on substrate metabolism, energy expenditure and body composition. In addition, if calcium did influence these factors during long duration exercise, then its impact on endurance performance would be quantified.

From the first exploratory study of heterogeneous mixed athletic sample it was shown that calcium supplementation increased total energy expenditure but did not have a statistically significant impact on fat metabolism. Plasma NEFA exhibited trends of increased appearance in the plasma with a concurrent trend in increased fat oxidation during a 60 min steady state (50% $\dot{V}O_{2peak}$) protocol (aim 1). In addition the body composition of the participants changed favourably with a reduction in fat mass and an increase in lean mass (aim 2). This was exhibited by the well-trained participants but this trend was reversed in the recreationally trained participants.

Thus the further 2 studies investigating the role of calcium on endurance performance were conducted on two varying durations, twenty five mile and ten mile

time trials. These tests were conducted on highly trained athletes based on the results from study 1. The results from these studies indicate that calcium supplementation improved power output and GE of the riders (aim 3) reducing the time to complete the trials. This improvement was however, more pronounced in the 25TT than in the 10TT. In addition the body composition of the participants showed a small but statistically non-significant decrease in body fat over a four week period.

Thus the involvement of calcium in performance enhancement warrants further investigation. Also, the mechanistic route or routes in which calcium intake is helpful to athletes in improving performance remains to be elucidated. As explored earlier, this may be due to various factors including attenuation of fatigue, improved lipolysis and vascular tone in addition to favourable changes in body composition. Thus, future studies designed to understand these mechanisms will be important to enhance our understanding of the role of calcium in energy metabolism. On the whole, benefits of understanding the complex role of calcium in energy metabolism range from amelioration of the spread of obesity, hypertension, diabetes and other areas of the metabolic syndrome to potential ergogenic effects on performance.

8.2 Future directions

From the original tap-water "incidence" in Sydney Ringer's laboratory (Ringer, 1883, Ringer, 1882) to the identification of the far reaching effects of changes to cellular ion handling in the development of hypertension, obesity, diabetes, cardiovascular abnormalities, the research in the area of the role of calcium ion in metabolism has come a long way. But, in many ways this is only scratching the surface. This investigation began with a simple hypothesis based on the negative

correlation between dietary calcium and body mass/fat; but the journey has been daunting in the sheer complexity and delicacy of the interactions between various cellular fractions and indeed entire systems within the body. The results of these studies demand further investigations in various aspects of the role of calcium metabolism; from lipolysis in the adipocytes (perhaps with direct measurements on rate of glycerol appearance) and IMTG in the skeletal muscle, insulin action on glucose transport and utilization, fatigue attenuation mechanisms, vascular tone, core body temperature via changes in UCP regulation, production of ROS and indeed changes in gross energy balance over time. Specifically UCP3 expressed in skeletal muscle that may have an involvement in regulation of ROS, in mitochondrial NEFA transport and in regulation of glucose metabolism in skeletal muscle, in addition to the role they play in energy regulation (Schrauwen and Hesselink, 2002). Also, recently, it has been shown that cycling efficiency is inversely proportional to the UCP3 content in skeletal muscle (Mogensen et al., 2006). With the involvement of calcium in the regulation of UCP content in both adipocyte and skeletal muscle (Sun and Zemel, 2004) this is a promising avenue to explore with regard to calcium an its role in energy partitioning and this body composition.

Some of the immediate and pertinent questions that arise from this investigation are perhaps the specific issue of the use of calcium as an ergogenic aid in sport. There is a need to tease out the mechanistic links between the increased power output and the relationship with improved GE and indeed performance. This investigation has only revealed a possible link between calcium and such factors such as increased power output with a few suggestive ideas on the underlying mechanisms. There needs to be specific studies designed to identify them in isolation. In addition, optimum dosage, timing, length of supplementation, differing levels of total energy intake along with

different macro nutrient combinations, interaction of other dietary micro nutrients aiding calcium or hindering its effect as an ergogenic aid, effects of long term habitual calcium intake on a short term high calcium supplementation program, different protocols with acute calcium loading and perhaps numerous as such need to be addressed before we can conclusively and confidently say more about the place of calcium on the sport nutrition podium.

Another interesting line of investigation would be the interaction of calcium used as an ergogenic aid with other well established ergogenic aids, for example caffeine. Caffeine is known to cause an increase in urinary excretion of calcium (Heaney and Recker, 1982), this as discussed in Chapter 2 has far reaching effects on the athletes, including increased peripheral vascular resistance and increased secretion of calcitropic hormones that may in turn cause the metabolic shift in substrate oxidation. However, when studying the ergogenic effects of caffeine per se, its role in CNS stimulation (Nehlig et al., 1992) which may involve changes in $_{i}[Ca^{2+}]$ levels (Allen and Westerblad, 1995), inhibition of phosphodiesterases (Nehlig and Debry, 1994) and blocking of cAMP (Nehlig and Debry, 1994, Nehlig et al., 1992) all of which may in turn influence or at least impact the effect calcium may have on performance. Caffeine has also shown to directly affect force production and raise $_{i}[Ca^{2+}]$ levels in mouse skeletal muscle (Allen and Westerblad, 1995Allen and Westerblad, 1995). Thus, such interactions will need closer inspection considering the wide spread use of substances such as caffeine in sport.

Also, the studies on fat metabolism, fat excretion and energy balance all need further investigation with the specific sporting populations, as there is increasing data on sedentary population but knowing the changes in physiological adaptations that occur in trained individuals these questions that have gained recognition in the

metabolically disturbed population need to be revisited within sporting populations. Finally, these questions will undoubtedly need to be readdressed with advances in technology and instrumentation design and thus create avenues that have not even been conceived of by the author.

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Appendix A

%CV haematological variables

FFA

Assay (NEFA)	Absorbance (Blank 1)	Absorbance (Blank 2)	Absorbance (Blank 3)	mean absorbance	StdDev	%CV
Study 1- Pl1	0.051	0.053	0.051	0.052	0.001	2.23
Study 1- Pl2	0.056	0.055	0.055	0.055	0.001	1.04
Study 1- Ca1	0.052	0.058	0.057	0.056	0.003	5.77
Study 1- Ca2	0.061	0.058	0.061	0.060	0.002	2.89
10TT Pl1	0.058	0.057	0.057	0.057	0.001	1.01
10TT Pl2	0.064	0.065	0.059	0.063	0.003	5.13
25TT Pl1	0.063	0.063	0.059	0.062	0.002	3.74
25TT Pl2	0.063	0.063	0.065	0.064	0.001	1.81
10TT Ca1	0.061	0.061	0.062	0.061	0.001	0.94
10TT Ca2	0.065	0.064	0.064	0.064	0.001	0.90
25TT Ca1	0.061	0.067	0.068	0.065	0.004	5.79
25TT Ca2	0.061	0.062	0.060	0.061	0.001	1.64
Inter assay				0.062	0.003	4.69

iPTH

Assay (iPTH)	Absorbance	Absorbance	mean absorbance	StdDev	%CV
Study 1- Pl1	2.25	2.22	2.23	0.02	0.95
Study 1- Pl2	2.18	2.09	2.13	0.06	2.98
Study 1- Ca1	2.28	2.26	2.27	0.01	0.62
Study 1- Ca2	1.83	1.94	1.88	0.07	4.13
Inter assay			2.16	0.14	6.81

Total calcium

Assay (total plasma calcium)	Absorbance	Absorbance	mean absorbance	StdDev	%CV
Study 1- Pl1	2.62	2.63	2.62	0.007	0.27
Study 1- Pl2	2.67	2.67	2.67	0	0.0
Study 1- Ca1	2.62	2.60	2.61	0.014	0.54
Study 1- Ca2	2.75	2.77	2.76	0.014	0.51
Inter assay			2.66	0.06	2.36

TAG

Assay (TAG)	Absorbance	Absorbance	mean absorbance	StdDev	%CV
Study 1- Pl1	0.877	0.875	0.876	0.001	0.16
Study 1- Pl2	0.853	0.850	0.851	0.002	0.25
Study 1- Ca1	0.852	0.855	0.853	0.002	0.25
Study 1- Ca2	0.869	0.87	0.869	0	0.08
Inter assay			0.862	0.011	1.30

Glucose

Assay (glucose)	Absorbance	Absorbance	mean absorbance	StdDev	%CV
Study 1- Pl1	4.51	4.56	4.53	0.039	0.87
Study 1- Pl2	4.46	4.47	4.46	0.005	0.13
Study 1- Ca1	4.55	4.55	4.55	0.003	0.08
Study 1- Ca2	4.40	4.41	4.40	0.005	0.13
Inter assay			4.48	0.063	1.39

RESEARCH ON THE ERGOGENIC PROPERTIES OF CALCIUM IN ENDURANCE EVENTS

Aim of the project – Is to investigate if calcium supplementation improves performance.
Rationale – There is mounting evidence that calcium rich diets promote the cellular processes that increase fat metabolism and thus spare glycogen during a training session which could delay fatigue in endurance type events.
A calcium rich diet over a long period of time helps to keep fat mass low.
Tests conducted – When an athlete agrees to participate in the study he/she will undergo a few tests which would include determination of –

1. **maximal oxygen uptake ($\dot{V}O_{2max}$)**
2. **maximum heart rate**
3. **peak power output**
4. **aerobic threshold**
5. **anaerobic threshold / Lactate threshold**
6. **body composition**
7. **Nutritional intake analysis.**
8. **Breakdown of percentage of carbohydrate and fat usage during a particular training session of set intensity.**

The results of the fitness tests and nutrition assessment can be used to determine your strengths and weaknesses during training.

This information can be used to provide specific training advice and guidance to **improve your $\dot{V}O_{2max}$, reduce sub-maximal $\dot{V}O_2$, improve peak power, enhance recovery, allowing you to train smarter** and ultimately improve your training and racing performance.

All of the above mentioned tests in a **commercial set up could cost in excess of £200.** These test results will be provided for **free** and any guidance required on training zones and/or nutritional strategies can be provided to individual athletes according to their **personal** goals and requirements.

Typical schedule for participating athletes –

- Participants will be asked to take either a **calcium supplement of 1000 mg** or a placebo for a period of **4 weeks** throughout the testing phase.
- Total commitment of **4 lab visits** in a period of 3 months
- The exercise test will include –
    - 1 $\dot{V}O_{2max}$
    - 3 tests of **60 min** duration at 50% of $\dot{V}O_{2max}$
- Tests will include –
    - Expired gas collection.
    - Capillary and venous blood sampling.

Appendix C

RESEARCH ON THE ERGOGENIC PROPERTIES OF CALCIUM IN ENDURANCE EVENTS

Basic structure of the trial –

One of two sets of supplements –

Placebo or calcium citrate 1000 mg/day for 30 days each in a random order.

Three tests –

- **Max power** – BCF protocol (start W/min according to weight and rider category with 20 W/min increment until voluntary exhaustion)
- **10-mile time trial** – quickest time to complete.
- **25-mile time trial** – quickest time to complete.

Tests will be conducted on 2 occasions. Before and after 30 days of supplementation.

Heart rate and oxygen consumption will be recorded throughout using an online Spirometer. These will then be used to calculate Anaerobic/ Aerobic thresholds along with blood lactate data.

Blood analysis –

- Lactate every min in Max power test
- Time trial – 1 capillary sample every 5 or 20 min until end of test. 2 min recovery sample until 10 min post cessation of test.

Dietary and activity record –

- Participants will be required to keep a diet diary of a typical week and weekend which will then be analysed for major nutrients, energy and calcium intakes.
- Participants will also need to supply a general log of training over the intervention to suitably account for any training related changes.

For further details please contact Rehana Jawadwala on 01772 892483 or 07737874450. Alternatively email on rjawadwala@uclan.ac.uk

Appendix D

University of Central Lancashire Health Questionnaire

UCLan Sports Science Labs: Health Screen Questionnaire

Name_____ **Age**_____ **Gender** **M** **F**

Address_____

_____ **Phone**_____

Height_____ **Weight**_____ **Date of test** _____

Profession_____

Stage 1 - Known Diseases (Medical Conditions)

1. List the medications you take on a regular basis.

2. Do you have diabetes? No Yes
   a) if yes, please indicate if it is insulin-dependent diabetes mellitus (IDDM) or non-insulin-dependent diabetes mellitus (NIDDM). IDDM NIDDM
   b) if IDDM, for how many years have you had IDDM? _____ years

3. Have you had a stroke? No Yes

4. Has your doctor ever said you have heart trouble? No Yes

5. Do you take asthma medication? No Yes

6. Are you or do you have reason to believe you may be pregnant? No Yes

7. Is there any other physical reason that prevents you from participating in an exercise program (e.g. cancer; osteoporosis; severe arthritis; mental illness; thyroid, kidney or liver disease)? No Yes

Stage 2 - Signs and Symptoms

8. Do you often have pains in your heart, chest, or surrounding areas, especially during exercise? No Yes

9. Do you often feel faint or have spells of severe dizziness during exercise? No Yes

10. Do you experience unusual fatigue or shortness of breath at rest or with mild exertion? No Yes

11. Have you had an attack of shortness of breath that came on after you stopped exercising? No Yes

12. Have you been awakened at night by an attack of shortness of breath? No Yes

13. Do you experience swelling or accumulation of fluid in or around your ankles? No Yes

14. Do you often get the feeling that your heart is beating faster, racing, or skipping beats, either at rest or during exercise? No Yes

15. Do you regularly get pains in your calves and lower legs during exercise which are not due to soreness or stiffness? No Yes

16. Has your doctor ever told you that you have a heart murmur? No Yes

Stage 3 - Cardiac Risk Factors

17. Do you smoke cigarettes daily, or have you quit smoking within the past two years? No Yes

    If yes, how many cigarettes per day do you smoke (or did you smoke in the past two years)? _____ per day

18. Has your doctor ever told you that you have high blood pressure? No Yes

19. Has your father, mother, brother, or sister had a heart attack or suffered from cardiovascular disease before the age of 65? No Yes
    If yes,
    a) Was the relative male or female? _____
    b) At what age did he or she have the stroke or heart attack? _____
    c) Did this person die suddenly as a result of the stroke or heart attack? No Yes

20. Have you experienced menopause before the age of 45? No Yes
    If yes, do you take hormone replacement medication? No Yes

If known, enter blood pressure and blood lipid values:

21. What is your systolic blood pressure? _____mmHg

22. What is your diastolic blood pressure? _____mmHg

23. What is your serum cholesterol level? _____mmol/L or mg/dL

24. What is your serum HDL level? _____mmol/L or mg/dL

25. What is your serum triglyceride level? _____mmol/L or mg/dL

Stage 4 - Exercise Intentions

26. Does your job involve sitting for a large part of the day? No Yes

27. What are your current activity patterns?
    a) Frequency: _____exercise sessions per week
    b) Intensity: Sedentary Moderate Vigorous
    c) History: <3 months 3-12 months >12 months
    d) Duration: _____minutes per session

28. What types of exercises do you do?

29. Do you want to exercise at a moderate intensity (e.g. brisk walking) or at a vigorous intensity (e.g. jogging)? Moderate Vigorous

I acknowledge that the above information is correct to the best of my knowledge.

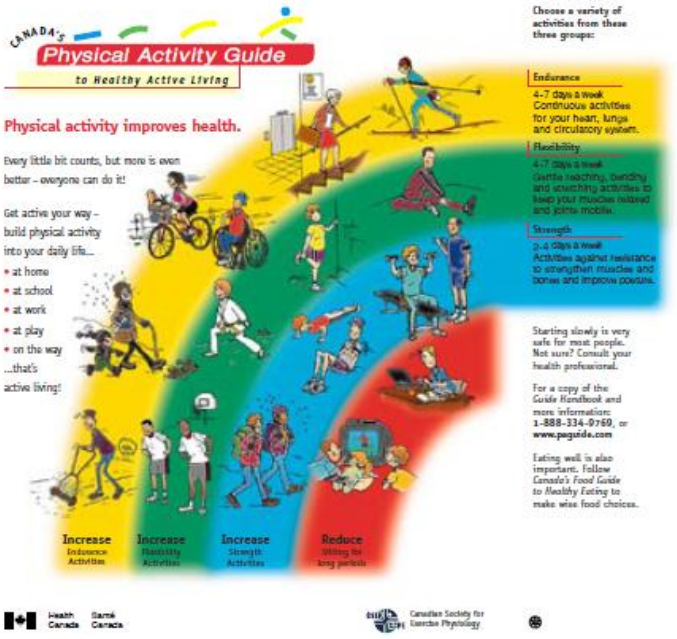
Sign: _____ Date: _____

Appendix E

...continued from other side

PAR-Q & YOU

Physical Activity Readiness Questionnaire - PAR-Q (revised 2002)



Choose a variety of activities from these three groups:

Endurance
4-7 days a week
Continuous activities for your heart, lungs and circulatory system.

Flexibility
4-7 days a week
Gentle reaching, bending and stretching activities to keep your muscles relaxed and joints mobile.

Strength
2-4 days a week
Activities against resistance to strengthen muscles and bones and improve posture.

Starting slowly is very safe for most people. Not sure? Consult your health professional.

For a copy of the *Guide Handbook* and more information: **1-888-334-9769**, or **www.paguide.com**

Eating well is also important. Follow *Canada's Food Guide to Healthy Eating* to make wise food choices.

Get Active Your Way, Every Day – For Life!

Scientists say accumulate 60 minutes of physical activity every day to stay healthy or improve your health. As you progress to moderate activities you can cut down to 30 minutes, 4 days a week. Add-up your activities in periods of at least 10 minutes each. Start slowly... and build up.

Time needed depends on effort

Very Light Effort	Light Effort 60 minutes	Moderate Effort 30-60 minutes	Vigorous Effort 20-30 minutes	Maximum Effort
• Strolling • Dusting	• Light walking • Volleyball • Easy gardening • Stretching	• Brisk walking • Biking • Raking leaves • Swimming • Dancing • Water aerobics	• Aerobics • Jogging • Hockey • Basketball • Fast swimming • Fast dancing	• Sprinting • Karting

Range needed to stay healthy

You Can Do It – Getting started is easier than you think

Physical activity doesn't have to be very hard. Build physical activities into your daily routine.

- Walk whenever you can – get off the bus early, use the stairs instead of the elevator.
- Reduce inactivity for long periods, like watching TV.
- Get up from the couch and stretch and bend for a few minutes every hour.
- Play actively with your kids.
- Choose to walk, wheel or cycle for short trips.
- Start with a 10 minute walk – gradually increase the time.
- Find out about walking and cycling paths nearby and use them.
- Observe a physical activity class to see if you want to try it.
- Try one class to start – you don't have to make a long-term commitment.
- Do the activities you are doing now, more often.

Benefits of regular activity:	Health risks of inactivity:
- better health - improved fitness - better posture and balance - better self-esteem - weight control - stronger muscles and bones - feeling more energetic - relaxation and reduced stress - continued independent living in later life	- premature death - heart disease - obesity - high blood pressure - adult-onset diabetes - osteoporosis - stroke - depression - colon cancer

Health Canada Santé Canada

Canadian Society for Exercise Physiology

Source: Canada's Physical Activity Guide to Healthy Active Living, Health Canada, 1998 http://www.hc-sc.gc.ca/hppb/paguide/pdf/guideEng.pdf

FITNESS AND HEALTH PROFESSIONALS MAY BE INTERESTED IN THE INFORMATION BELOW:

The following companion forms are available for doctors' use by contacting the Canadian Society for Exercise Physiology (address below):

The **Physical Activity Readiness Medical Examination (PARmed-X)** – to be used by doctors with people who answer YES to one or more questions on the PAR-Q.

The **Physical Activity Readiness Medical Examination for Pregnancy (PARmed-X for Pregnancy)** – to be used by doctors with pregnant patients who wish to become more active.

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References:

Arraix, G.A., Wigle, D.T., Mao, Y. (1992). Risk Assessment of Physical Activity and Physical Fitness in the Canada Health Survey Follow-Up Study. **J. Clin. Epidemiol.** 45:4 419-428.

Mottola, M., Wolfe, L.A. (1994). Active Living and Pregnancy. In: A. Quinney, L. Gauvin, T. Wall (eds.), **Toward Active Living: Proceedings of the International Conference on Physical Activity, Fitness and Health**. Champaign, IL: Human Kinetics.

PAR-Q Validation Report, British Columbia Ministry of Health, 1978.

Thomas, S., Reading, J., Shephard, R.J. (1992). Revision of the Physical Activity Readiness Questionnaire (PAR-Q). **Can. J. Spt. Sci.** 17:4 338-345.

For more information, please contact the:

Canadian Society for Exercise Physiology
202-185 Somerset Street West
Ottawa, ON K2P 0J2
Tel. 1-877-651-3755 • FAX (613) 234-3565
Online: www.csep.ca

The original PAR-Q was developed by the British Columbia Ministry of Health. It has been revised by an Expert Advisory Committee of the Canadian Society for Exercise Physiology chaired by Dr. N. Gledhill (2002).

Disponible en français sous le titre «Questionnaire sur l'aptitude à l'activité physique - Q-AAP (revisé 2002)».

Supported by: Health Canada Santé Canada

Physical Activity Readiness Questionnaire - PAR-Q (revised 2002)

PAR-Q & YOU

(A Questionnaire for People Aged 15 to 69)

Regular physical activity is fun and healthy, and increasingly more people are starting to become more active every day. Being more active is very safe for most people. However, some people should check with their doctor before they start becoming much more physically active.

If you are planning to become much more physically active than you are now, start by answering the seven questions in the box below. If you are between the ages of 15 and 69, the PAR-Q will tell you if you should check with your doctor before you start. If you are over 69 years of age, and you are not used to being very active, check with your doctor.

Common sense is your best guide when you answer these questions. Please read the questions carefully and answer each one honestly: check YES or NO.

YES	NO		
☐	☐	1.	**Has your doctor ever said that you have a heart condition and that you should only do physical activity recommended by a doctor?**
☐	☐	2.	**Do you feel pain in your chest when you do physical activity?**
☐	☐	3.	**In the past month, have you had chest pain when you were not doing physical activity?**
☐	☐	4.	**Do you lose your balance because of dizziness or do you ever lose consciousness?**
☐	☐	5.	**Do you have a bone or joint problem (for example, back, knee or hip) that could be made worse by a change in your physical activity?**
☐	☐	6.	**Is your doctor currently prescribing drugs (for example, water pills) for your blood pressure or heart condition?**
☐	☐	7.	**Do you know of any other reason why you should not do physical activity?**

If you answered YES to one or more questions

Talk with your doctor by phone or in person BEFORE you start becoming much more physically active or BEFORE you have a fitness appraisal. Tell your doctor about the PAR-Q and which questions you answered YES.

- You may be able to do any activity you want — as long as you start slowly and build up gradually. Or, you may need to restrict your activities to those which are safe for you. Talk with your doctor about the kinds of activities you wish to participate in and follow his/her advice.
- Find out which community programs are safe and helpful for you.

NO to all questions

If you answered NO honestly to all PAR-Q questions, you can be reasonably sure that you can:

- start becoming much more physically active – begin slowly and build up gradually. This is the safest and easiest way to go.
- take part in a fitness appraisal – this is an excellent way to determine your basic fitness so that you can plan the best way for you to live actively. It is also highly recommended that you have your blood pressure evaluated. If your reading is over 144/94, talk with your doctor before you start becoming much more physically active.

DELAY BECOMING MUCH MORE ACTIVE:

- if you are not feeling well because of a temporary illness such as a cold or a fever – wait until you feel better; or
- if you are or may be pregnant – talk to your doctor before you start becoming more active.

PLEASE NOTE: If your health changes so that you then answer YES to any of the above questions, tell your fitness or health professional. Ask whether you should change your physical activity plan.

Informed Use of the PAR-Q: The Canadian Society for Exercise Physiology, Health Canada, and their agents assume no liability for persons who undertake physical activity, and if in doubt after completing this questionnaire, consult your doctor prior to physical activity.

No changes permitted. You are encouraged to photocopy the PAR-Q but only if you use the entire form.

NOTE: If the PAR-Q is being given to a person before he or she participates in a physical activity program or a fitness appraisal, this section may be used for legal or administrative purposes.

"I have read, understood and completed this questionnaire. Any questions I had were answered to my full satisfaction."

NAME _____

SIGNATURE _____ DATE _____

SIGNATURE OF PARENT or GUARDIAN (for participants under the age of majority) _____ WITNESS _____

Note: This physical activity clearance is valid for a maximum of 12 months from the date it is completed and becomes invalid if your condition changes so that you would answer YES to any of the seven questions.

Supported by: Health Canada Santé Canada

continued on other side...

Appendix F

FREE AND INFORMED CONSENT

This study will involve a maximal oxygen uptake test and subsequent cycle ergometer exercise program set at 50% of your maximum power output for duration of 60 min. You will be required to perform 4 such cycle ergometer exercise sessions over a period of 3 months.

Capillary blood samples will be drawn every 20 min during each of the exercise sessions. One venous blood sample will be drawn at the beginning of each test.

You will be required to supplement your normal diet with 1000 mg of calcium citrate and placebo every day for a period of 4 weeks, with a month break in between the two trials. If you experience any discomfort during the period related to the calcium supplement or placebo, please inform the researcher as soon as possible.

Body composition will be determined using the Harpenden callipers.

You will be required to maintain a diet record for seven consecutive days.

All the tests will be explained to you prior to commencement. During this time you will have an opportunity to ask any questions about the test and are free to withdraw at any time.

All the tests are performed in such a way as to minimise any risk of injury. Please inform the researcher of any reason why you should not perform exercise. If at any time you feel undue pain or discomfort, stop the test and inform the researcher of your symptoms.

The tests and measures have been explained to me and I am willing to take part. I understand that I can withdraw at any time.

Signed: _____

Witness: _____

Date: _____

Appendix G

FREE AND INFORMED CONSENT

This study aims to look at the effects of calcium supplementation on endurance performance.

The experiment involves one maximal power ramp test followed by two time trials of 10 mile and 25 mile. Both the time trials will be conducted before and after the intervention.

Capillary blood samples will be drawn every 5 min in the 10 mile time trial and every 20 min during the 25 mile time trial. In addition, lactate measurements will be continued to be taken after the test every 2.5 min for 10 min.

You will be required to supplement your normal diet with 1000 mg/day of calcium citrate or placebo for a period of 4 weeks. If you experience any discomfort during the period related to the calcium supplement or placebo, please inform the researcher as soon as possible.

Body composition will be determined using the Harpenden callipers and BodPod.

You will be required to maintain a diet and physical activity record for seven consecutive days.

All the tests will be explained to you prior to commencement. During this time you will have an opportunity to ask any questions about the test and are free to withdraw at any time.

All the tests are performed in such a way as to minimise any risk of injury. Please inform the researcher of any reason why you should not perform exercise. If at any time you feel undue pain or discomfort, stop the test and inform the researcher of your symptoms.

The tests and measures have been explained to me and I am willing to take part. I understand that I can withdraw at any time.

Signed: _____

Witness: _____

Date: _____

Appendix H

UNIVERSITY OF CENTRAL LANCASHIRE

Centre for Applied Sports and Exercise Science
School of Psychology

Food Diary

The aim of this diary is to help you keep an accurate record of everything you eat and drink over a seven day period. This will help enable us to determine dietary nutrient intake.

If you have any problems or queries regarding this diary, please feel free to contact Rehana (01772) 892483 or email rjawadwala@uclan.ac.uk

CHECKLIST

Given below are a list of commonly eaten foods. Please tick the kind(s) normally used.

Milk	Whole	☐	Semi-skinned	☐	Skimmed	☐	Other	☐
Cheese	Cream	☐	Hard	☐	Low-fat	☐	Other	☐
Yoghurt	Full-fat	☐	Low-fat	☐	Diet	☐	Other	☐
Bread	White	☐	Wholemeal	☐	Hovis/brown	☐	Other	☐
Spread	Butter	☐	Low-fat	☐	Margarine	☐	Other	☐

DO YOU	**Yes**	**No**	**If yes, please give amount**
Take milk in tea or coffee?	☐	☐	☐
Take sugar in tea or coffee?	☐	☐	☐
Use jam, honey or marmalade?	☐	☐	☐
Drink alcohol?	☐	☐	☐

FOOD DESCRIPTIONS

Please ensure you include the following points in the food description category.

1) Fruit. Is it raw or cooked, fresh or tinned. If tinned, is it in syrup or juice.
2) Vegetables. Raw or cooked, fresh, frozen or tinned. In sauce.
3) Meat, poultry and fish. Have you weighed as raw or cooked. Has the skin or fat been removed before weighing.
4) Gravy. Has meat juice or fat been added.
5) Mayonnaise and dressings. Full or low-fat. Include brand name.

Participant I.D. Number …… Participant Initials …… Diary Number ……..

Day ……

Meal	Food Description	Cooking Method	Weight in grams	Amount Uneaten
Breakfast				
During the Morning				
Lunch				

Meal	Food Description	Cooking Method	Weight in grams	Amount Uneaten
During the afternoon				
Dinner or Tea				
During the evening				

Kevin Gibbons

Training:-
20K on static bike - Low resist high reps - 35 mins. Core stability exercises

Subject I.D. Number ..... **Subject Initials** KRG **Diary Number** .....

Day Mon. 10th Nov

Meal	Food Description	Cooking Method	Weight in grams	Amount Uneaten
Breakfast	Fruit Salad (RAW)	Cold	Medium	all
	Wheatabix & Museli + banana in skimmed milk	Hot	Large	
	Slice of Wholemeal toast 2 Thin slices of Malt Loaf with Peanut butter		Medium Small	
During the Morning	Homemade energy cake (oats, raisins, mixed mango, peanut butter & honey)	Baked	Medium	
Lunch	3 slices of wholemeal bread with Humous & red pepper	Cold	Medium	
	½ raw carrot 1 tomato		Small Small	

Appendix I

Mean (± SD) Energy, macronutrient, calcium and vitamin D consumption based on seven day diet diaries recorded during each of the 4 week intervention period in study 1

	Pl Mean ± SD **(%Recommendations)**	**Ca** Mean ± SD **(%Recommendations)**	Recommendations*
Energy (MJ/d)	8.3 ± 1.5	8.3 ± 1.3	10.60 males 8.10 females
Energy (Kcal/d)	1995 ± 368	1984 ± 330	2,550 males 1,940 females
Total carbohydrates (g/d)	259 ± 72 **(52 ± 14% EI)**	257 ± 68 **(52 ± 14% EI)**	47% EI
Non milk extrinsic sugars (g/d)	65.10 ± 16.41 **(11.59 ± 2.92% EI)**	62.70 ± 17.80 **(11.16 ± 3.17% EI)**	10% EI
Non starch polysaccharide (g/d)	11.51 ± 3.41 **(63 ± 18.9% population mean)**	12.32 ± 3.43 **(68.44 ± 19.05% population mean)**	12 (individual min) 18 (population mean)
Total fat (g/d)	59 ± 8 **(27 ± 3% EI)**	60 ± 9 **(27 ± 4% EI)**	33% EI
Saturated fatty acids (g/d)	24.86 ± 7.57 **(9.96 ± 3.03% EI)**	24.93 ± 8.83 **(9.98 ± 3.53% EI)**	10% EI
Cis-polyunsaturated fatty acids (g/d)	16.77 ± 6.99 **(6.72 ± 2.80% EI)**	15.27 ± 8.00 **(6.11 ± 3.20% EI)**	6% EI
Cis-monounsaturated fatty acids (g/d)	20.32 ± 7.11 **(8.14 ± 2.85% EI)**	22.17 ± 8.69 **(8.88 ± 3.48% EI)**	12% EI
Protein (g/d)	85 ± 17 **(17 ± 3% EI)** **(~156 ± 45% RNI)**	84 ± 15 **17 ± 3% EI)** **(~155 ± 35% RNI)**	55.5 g/d males 45.0 g/d females
Calcium (mg/d)	770 ± 97 **(110 ± 14% RNI)**	772 ± 114 **(110 ± 16% RNI)**	700
Vitamin D (µg/d)	2.85 ± 1.34	2.80 ± 1.24	-

***Source: COMA-Committee on Medical Aspects of Food Policy (1991)**

Mean (± SD) Energy, macronutrient, calcium and vitamin D consumption based on seven day diet diaries recorded during the 4 week intervention period in study 2 and 3

	Mean ± SD **(%Recommendations)**	Recommendations*
Energy (MJ/d)	9.6 ± 1.4 **(90.56 ± 13.2% EAR)**	10.60
Energy (Kcal/d)	2309 ± 358 **(90.56 ± 13.2% EAR)**	2,550
Total carbohydrates (g/d)	334± 75 **(58 ± 12% EI)**	47% EI
Non milk extrinsic sugars (g/d)	89.00 ± 42.30 **(16.27 ± 7.73% EI)**	10% EI
Non starch polysaccharide (g/d)	12.50 ± 4.86 **(69 ± 27% population mean)**	12 (individual min) 18 (population mean)
Total fat (g/d)	60 ± 14 **(23 ± 5% EI)**	33% EI
Saturated fatty acids (g/d)	19.13 ± 5.19 **(7.86 ± 2.13% EI)**	10% EI
Cis-polyunsaturated fatty acids (g/d)	11.03 ± 4.88 **(4.53 ± 2.00% EI)**	6% EI
Cis-monounsaturated fatty acids (g/d)	18.03 ± 2.86 **(7.41 ± 1.17% EI)**	12% EI
Protein (g/d)	93 ± 23 **(16 ± 4% EI)** **(140 ± 13.45% RNI)**	55.5
Calcium (mg/d)	757 ± 108 **(108 ± 15% RNI)**	700
Vitamin D (µg/d)	2.92 ± 1.36	-

***Source: COMA-Committee on Medical Aspects of Food Policy (1991)**

Mean Energy, macronutrient and micronutrient consumption of each participant based on 7 d diet diaries recorded during each of the 4 week intervention periods in study 1.

R1	Pl	Ca			Pl	Ca	
	Mean ± SD	Mean ± SD	CV (%)		Mean ± SD	Mean ± SD	CV (%)
Energy (MJ)	5.8 ± 0.2	5.9 ± 0.2	0.41	MUFA (g)	12 ± 11	44 ± 28	4.29
Energy (kcal)	1408 ± 6	2696 ± 10	0.41	Protein (g)	70.6 ± 1.4	72.6 ± 1.7	1.98
Fat (g)	58.7 ± 0.5	58.0 ± 0.8	0.85	CHO (g)	123 ± 22	129 ± 17	3.37
SFA (g)	32.8 ± 3.2	26.4 ± 9.1	15.29	Sugars (g)	66 ± 19	53 ± 22	15.45
PUFA (g)	12 ± 4	6 ± 3	2.81	Ca (mg)	679 ± 24	713 ± 14	3.45

R2	Pl	Ca			Pl	Ca	
	Mean ± SD	Mean ± SD	CV (%)		Mean ± SD	Mean ± SD	CV (%)
Energy (MJ)	7.7 ± 0.3	8.2 ± 0.3	4.77	MUFA (g)	19.6 ± 6.7	25.4 ± 6.9	18.23
Energy (kcal)	1845 ± 90	1974 ± 77	4.77	Protein (g)	88.3 ± 5	86 ± 7	1.87
Fat (g)	55 ± 7	63 ± 1	9.72	CHO (g)	232 ± 18	243 ± 12	3.28
SFA (g)	27.3 ± 13	36.4 ± 24.3	20.2	Sugars (g)	69 ± 13	64 ± 11	4.84
PUFA (g)	9.7 ± 2	10.3 ± 4	4.24	Ca (mg)	947 ± 24	1002 ± 17	3.99

R3	Pl	Ca			Pl	Ca	
	Mean ± SD	Mean ± SD	CV (%)		Mean ± SD	Mean ± SD	CV (%)
Energy (MJ)	7.0 ± 0.6	7.1 ± 0.5	0.89	MUFA (g)	14 ± 2	8.9 ± 3.8	31.5
Energy (kcal)	1677 ±29	1698 ± 51	0.89	Protein (g)	74 ± 6	77 ± 8	2.81
Fat (g)	63 ± 5	58.7 ± 3	5.00	CHO (g)	204 ± 29	216 ± 12	4.05
SFA (g)	13.9 ± 3.3	13.6 ± 6.1	2.06	Sugars (g)	43 ± 16	48 ± 23	8.49
PUFA (g)	11.1 ± 10	13.8 ± 7	15.33	Ca (mg)	745 ± 8	734 ± 14	1.05

R4	Pl	Ca			Pl	Ca	
	Mean ± SD	Mean ± SD	CV (%)		Mean ± SD	Mean ± SD	CV (%)
Energy (MJ)	8.0 ± 0.35	7.5 ± 0.65	4.57	MUFA (g)	16 ± 4	14 ± 11	9.43
Energy (kcal)	1908 ± 89	1789 ± 71	4.57	Protein (g)	82.7 ± 22	80.5 ± 7	1.91
Fat (g)	67.2 ± 7	67.9 ± 12	0.73	CHO (g)	224 ± 18	200 ± 17	8.00
SFA (g)	25.6 ± 6.2	21.1 ± 12	13.96	Sugars (g)	74 ± 24	63 ± 8	11.35
PUFA (g)	22.1 ± 3	16.8 ± 6.6	19.27	Ca (mg)	687 ± 23	624 ± 39	6.80

T1	Pl	Ca			Pl	Ca	
	Mean ± SD	Mean ± SD	CV (%)		Mean ± SD	Mean ± SD	CV (%)
Energy (MJ)	8.2 ± 0.4	8.8 ± 0.3	4.97	MUFA (g)	12 ± 4	18 ± 3.8	28.28
Energy (kcal)	1976 ± 101	2120 ± 87	4.97	Protein (g)	79.3 ± 9	86.3 ± 3	5.98
Fat (g)	52.9 ± 16	50.80 ± 29	2.86	CHO (g)	287 ± 22	319 ± 35	7.47
SFA (g)	16.3 ± 4	14.1 ± 12	10.23	Sugars (g)	56 ± 27	55 ± 24	1.40
PUFA (g)	12.6 ± 10	14.3 ± 8	8.94	Ca (mg)	749 ± 31	760 ± 26	1.03

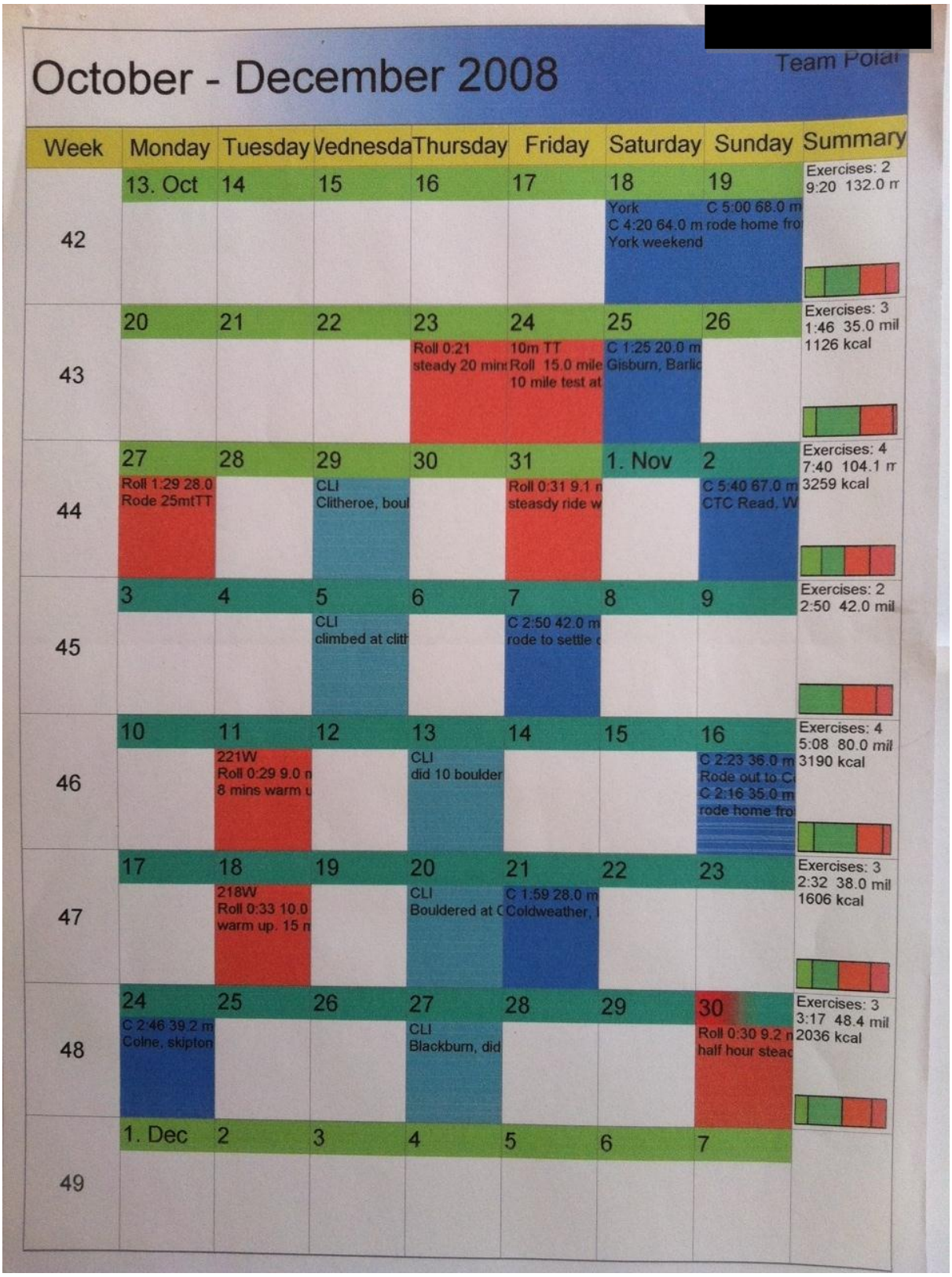
T2	Pl	Ca			Pl	Ca	
	Mean ± SD	Mean ± SD	CV (%)		Mean ± SD	Mean ± SD	CV (%)
Energy (MJ)	9.3 ± 0.2	8.9 ± 0.4	3.00	MUFA (g)	31 ± 5	34 ± 9	6.53
Energy (kcal)	2220 ± 126	2128 ± 177	3.00	Protein (g)	81 ± 26	78 ± 34	2.67
Fat (g)	44.7 ± 9	46 ± 12	2.03	CHO (g)	356 ± 111	340 ± 102	3.25
SFA (g)	34.1 ± 15	39.6 ± 10	10.55	Sugars (g)	94 ± 32	86 ± 24	6.29
PUFA (g)	31.1 ± 14	34 ± 17	6.53	Ca (mg)	781 ± 16	803 ± 31	1.96

T3	Pl	Ca			Pl	Ca	
	Mean ± SD	Mean ± SD	CV (%)		Mean ± SD	Mean ± SD	CV (%)
Energy (MJ)	9.0 ± 0.7	8.8 ± 0.7	0.8	MUFA (g)	19 ± 16	16 ± 11	12.12
Energy (kcal)	2150 ± 309	2126 ± 288	0.8	Protein (g)	72.1 ± 23	69.0 ± 16	3.11
Fat (g)	66 ± 7	69 ± 12	3.04	CHO (g)	289 ± 91	276 ± 122	3.25
SFA (g)	26.1 ± 4	21.7 ± 15	13.02	Sugars (g)	65 ± 10	61 ± 13	4.49
PUFA (g)	12.1 ± 6	10 ± 6	13.44	Ca (mg)	900 ± 67	887 ± 22	1.03

T4	Pl	Ca			Pl	Ca	
	Mean ± SD	Mean ± SD	CV (%)		Mean ± SD	Mean ± SD	CV (%)
Energy (MJ)	11.4 ± 0.7	10.8 ± 0.7	3.56	MUFA (g)	23 ± 14	26 ± 7	8.66
Energy (kcal)	2721 ± 123	2587 ± 201	3.56	Protein (g)	128 ± 36	120 ± 45	4.56
Fat (g)	69.4 ± 18	73 ± 12	3.58	CHO (g)	340 ± 113	324 ± 139	3.41
SFA (g)	31.1 ± 4.6	25.9 ± 19	12.9	Sugars (g)	42 ± 15	38 ± 13	7.07
PUFA (g)	16.4 ± 2	18.9 ± 5.2	10.02	Ca (mg)	670 ± 31	670 ± 43	0.00

T5	Pl	Ca			Pl	Ca	
	Mean ± SD	Mean ± SD	CV (%)		Mean ± SD	Mean ± SD	CV (%)
Energy (MJ)	8.6 ± 0.8	8.5 ± 0.7	0.89	MUFA (g)	32 ± 4	34 ± 1	4.29
Energy (kcal)	2052 ± 181	2026 ± 176	0.89	Protein (g)	88 ± 26	87 ± 21	0.73
Fat (g)	56.9 ± 9	58 ± 12	1.35	CHO (g)	276 ± 76	268 ± 61	2.08
SFA (g)	32.8 ± 15	26.4 ± 17	15.29	Sugars (g)	66 ± 14	55 ±13	15.45
PUFA (g)	22 ± 4	6 ± 12	80.81	Ca (mg)	769 ± 16	759 ± 24	0.93

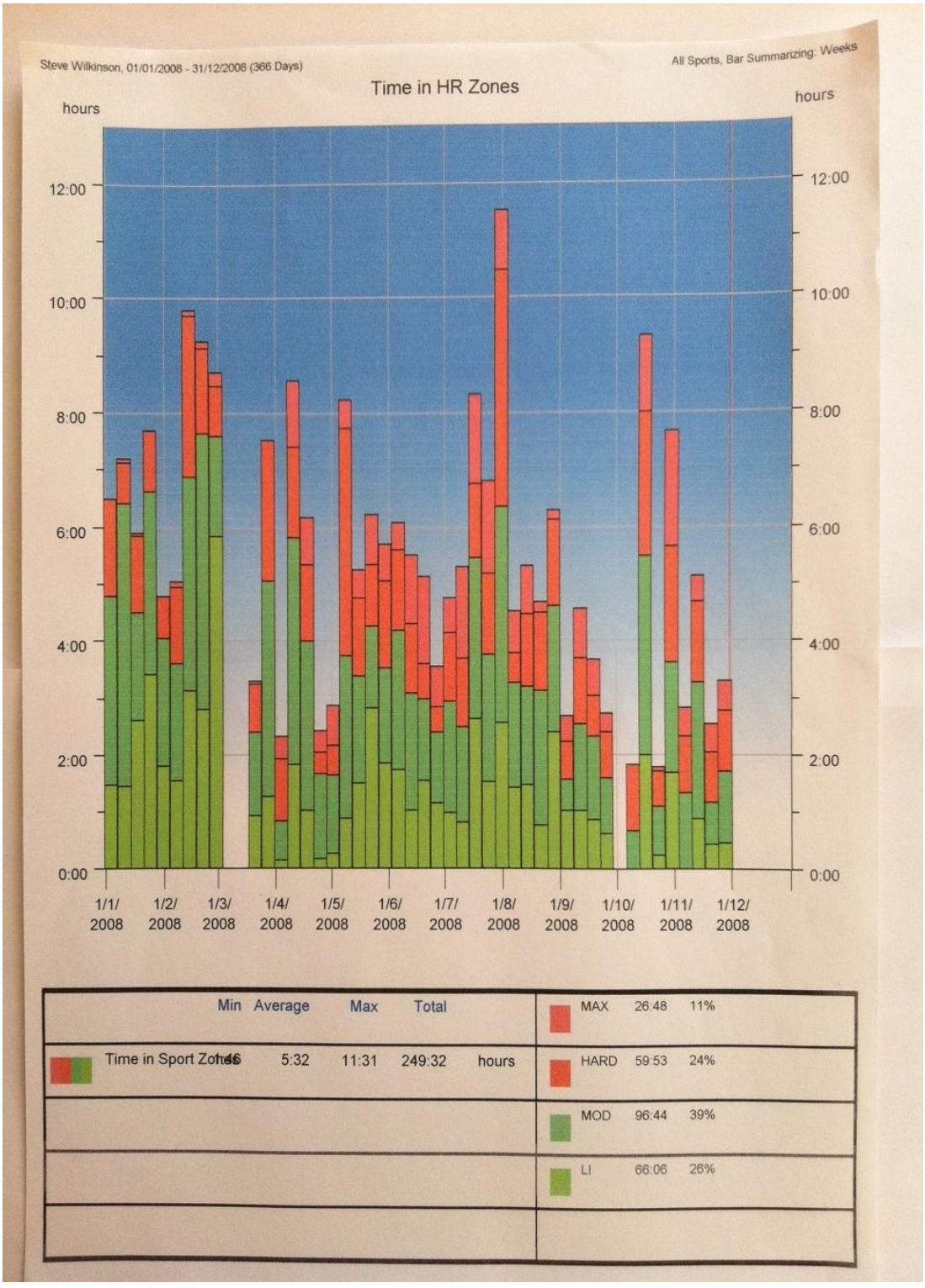
Appendix J

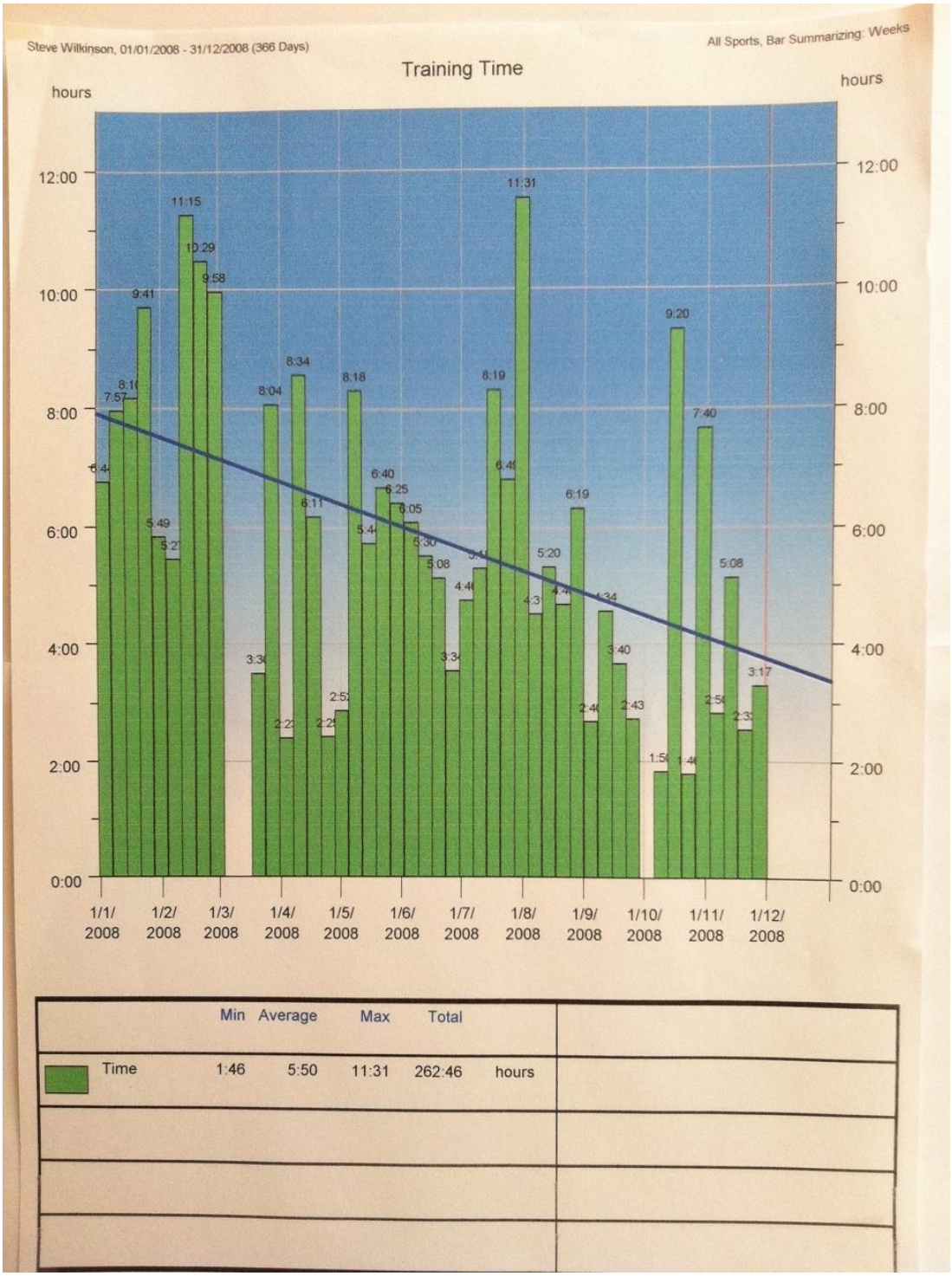


October - December 2008

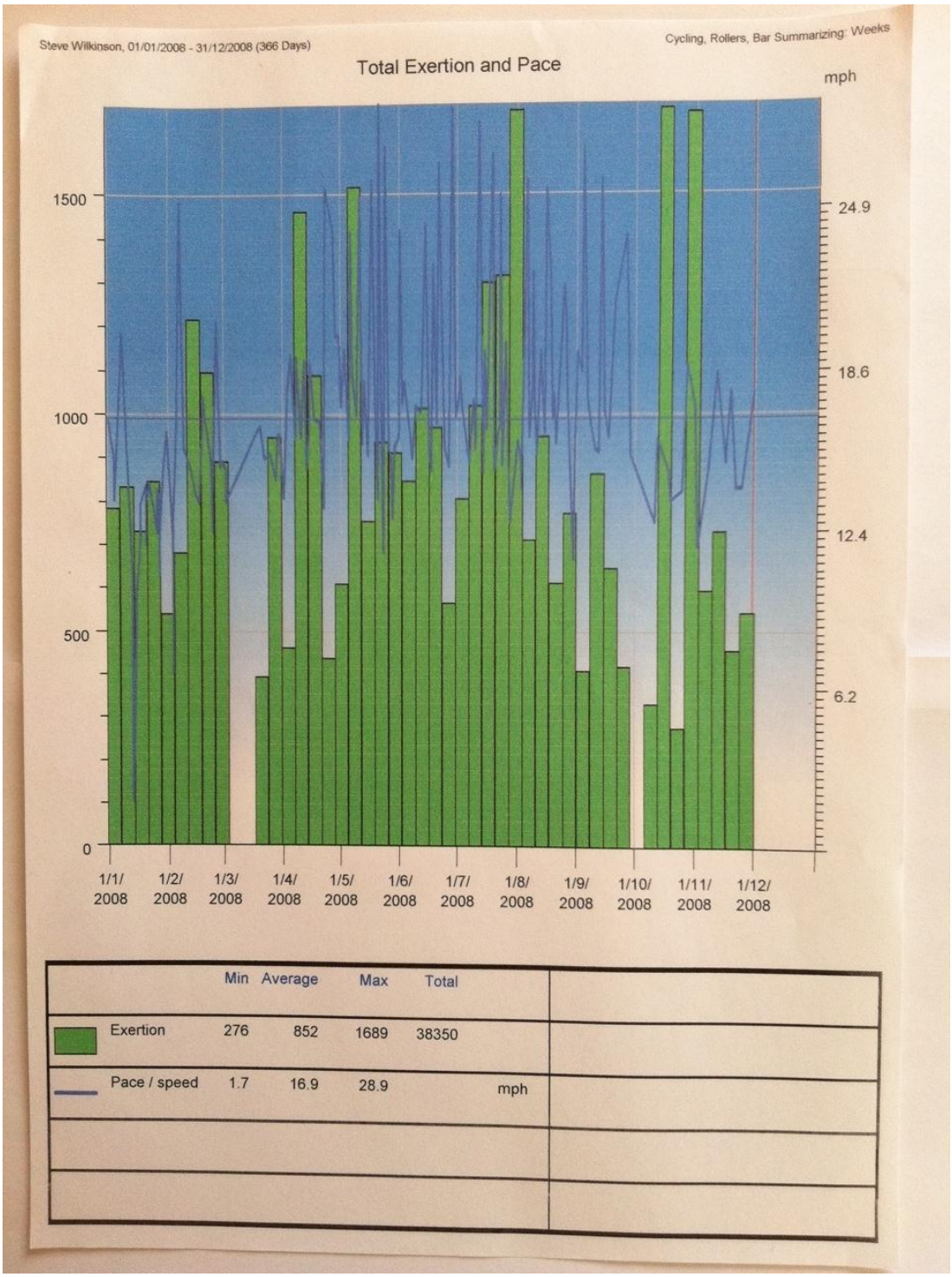
Team Polar

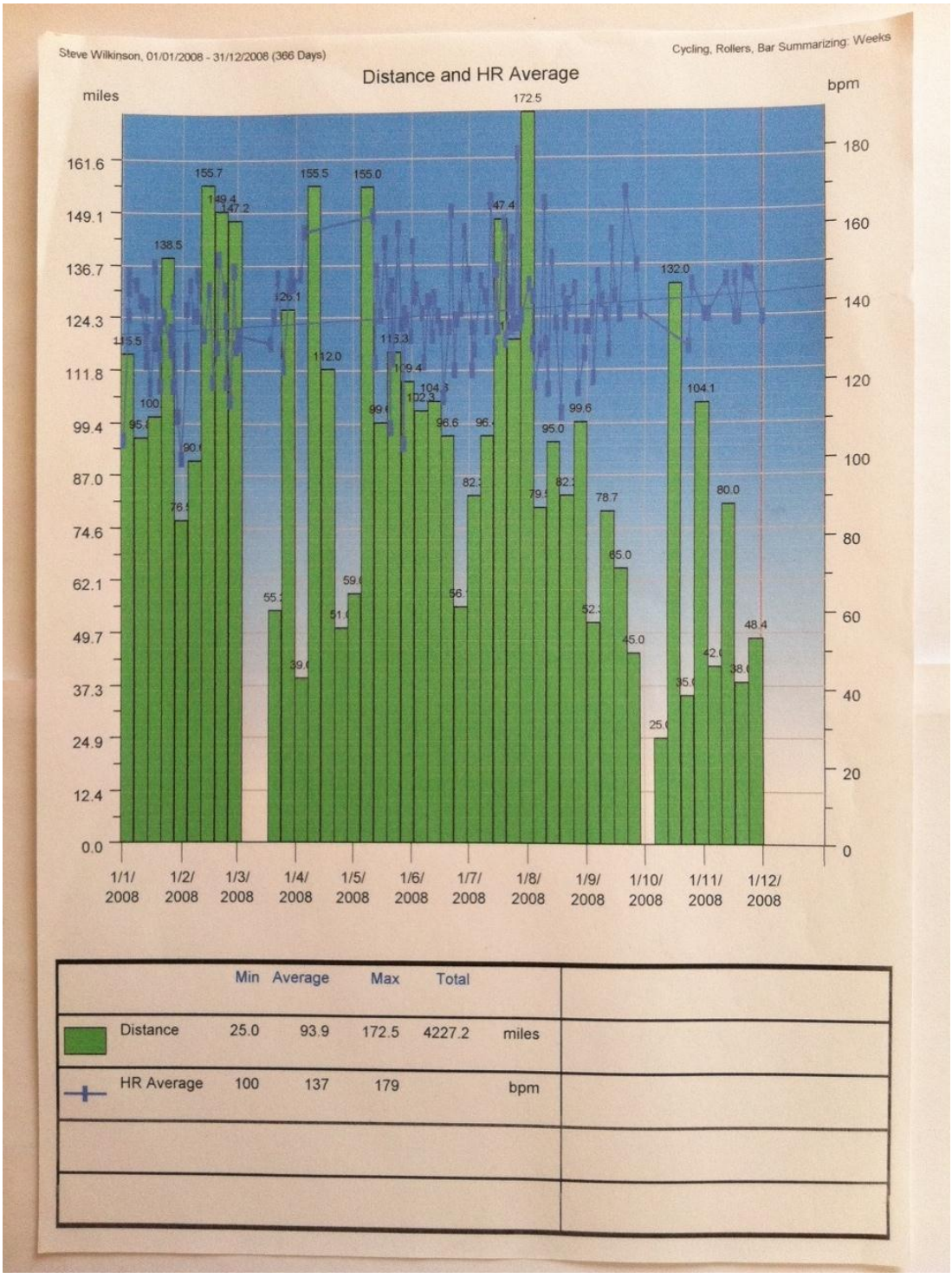
Week	Monday	Tuesday	Vednesda	Thursday	Friday	Saturday	Sunday	Summary
42	13. Oct	14	15	16	17	18 York C 4:20 64.0 m York weekend	19 C 5:00 68.0 m rode home fro	Exercises: 2 9:20 132.0 m
43	20	21	22	23 Roll 0:21 steady 20 min	24 10m TT Roll 15.0 mile 10 mile test at	25 C 1:25 20.0 m Gisburn, Barlic	26	Exercises: 3 1:46 35.0 mil 1126 kcal
44	27 Roll 1:29 28.0 Rode 25mtTT	28	29 CLI Clitheroe, boul	30	31 Roll 0:31 9.1 n steasdy ride w	1. Nov	2 C 5:40 67.0 m CTC Read. W	Exercises: 4 7:40 104.1 m 3259 kcal
45	3	4	5 CLI climbed at clith	6	7 C 2:50 42.0 m rode to settle	8	9	Exercises: 2 2:50 42.0 mil
46	10	11 221W Roll 0:29 9.0 n 8 mins warm u	12	13 CLI did 10 boulder	14	15	16 C 2:23 36.0 m Rode out to C C 2:16 35.0 m rode home fro	Exercises: 4 5:08 80.0 mil 3190 kcal
47	17	18 218W Roll 0:33 10.0 warm up. 15 n	19	20 CLI Bouldered at C	21 C 1:59 28.0 m Coldweather,	22	23	Exercises: 3 2:32 38.0 mil 1606 kcal
48	24 C 2:46 39.2 m Colne, skipton	25	26	27 CLI Blackburn, did	28	29	30 Roll 0:30 9.2 n half hour steac	Exercises: 3 3:17 48.4 mil 2036 kcal
49	1. Dec	2	3	4	5	6	7	


Steve Wilkinson, 01/01/2008 - 31/12/2008 (366 Days)
All Sports, Bar Summarizing: Weeks
Time in HR Zones
hours
hours
12:00
10:00
8:00
6:00
4:00
2:00
0:00
1/1/ 2008
1/2/ 2008
1/3/ 2008
1/4/ 2008
1/5/ 2008
1/6/ 2008
1/7/ 2008
1/8/ 2008
1/9/ 2008
1/10/ 2008
1/11/ 2008
1/12/ 2008
Min Average Max Total
Time in Sport Zones 46 5:32 11:31 249:32 hours
MAX 26:48 11%
HARD 59:53 24%
MOD 96:44 39%
LI 66:06 26%



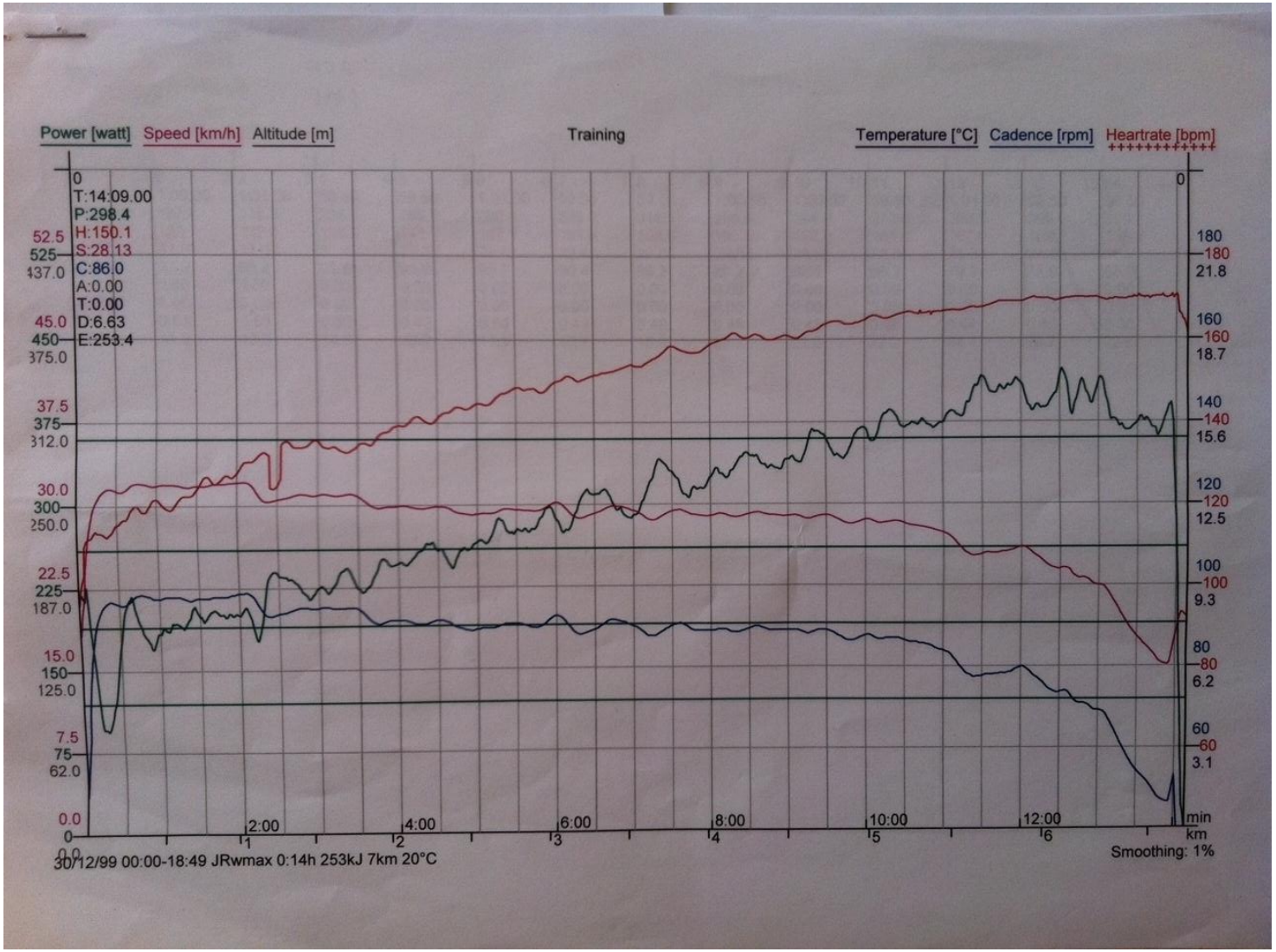
	Min	Average	Max	Total	
Time	1:46	5:50	11:31	262:46	hours


Steve Wilkinson, 01/01/2008 - 31/12/2008 (366 Days)
Cycling, Rollers, Bar Summarizing: Weeks
Total Exertion and Pace
mph
1500
1000
500
0
24.9
18.6
12.4
6.2
1/1/ 2008
1/2/ 2008
1/3/ 2008
1/4/ 2008
1/5/ 2008
1/6/ 2008
1/7/ 2008
1/8/ 2008
1/9/ 2008
1/10/ 2008
1/11/ 2008
1/12/ 2008
Min Average Max Total
Exertion 276 852 1689 38350
Pace / speed 1.7 16.9 28.9 mph



	Min	Average	Max	Total	
Distance	25.0	93.9	172.5	4227.2	miles
HR Average	100	137	179		bpm

Appendix K


Power [watt]
Speed [km/h]
Altitude [m]
Training
Temperature [°C]
Cadence [rpm]
Heartrate [bpm]
T:14:09.00
P:298.4
H:150.1
S:28.13
C:86.0
A:0.00
T:0.00
D:6.63
E:253.4
2:00
4:00
6:00
8:00
10:00
12:00
min
km
30/12/99 00:00-18:49 JRwmax 0:14h 253kJ 7km 20°C
Smoothing: 1%